University of Alberta

Arginine synthesis and metabolism in neonatal piglets

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science
Edmonton, Alberta
Spring, 2007

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Your file Votre référence
ISBN: 978-0-494-29757-5
Our file Notre référence
ISBN: 978-0-494-29757-5

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"*The future belongs to those who believe in the beauty of their dreams.*"

Eleanor Roosevelt

ABSTRACT

Arginine is an indispensable amino acid for neonates, because although they can synthesize some arginine, it is not enough to maintain health or support optimum growth. The main objective of this thesis was to study the sites and limitations of arginine synthesis in neonatal piglets. Five studies were conducted using a multi-catheterized piglet model, which allowed for tight control of dietary intake (either enteral or parenteral), isotope infusion and blood sampling. Stable and radioisotopes, infused intravenously, intragastrically or intraportally, were used to measure the conversion rates between arginine and its precursors proline, ornithine and citrulline. An arginine-deficient diet was used in all studies to create a metabolic state where there would be maximal stimulus for arginine synthesis.

In enterally-fed piglets, the conversion rate [μmol/(kg·h)] of circulating proline to arginine was greater in piglets receiving the arginine-deficient diet than in those receiving a generous intake of arginine (47 vs. 15; pooled SE 4) ($P < 0.05$); however, regardless of arginine intake, there was no arginine synthesis during first-pass hepatic metabolism. In parenterally-fed piglets receiving an arginine-deficient diet, the rate of arginine synthesis from circulating proline was directly related to mucosal mass ($R^2 = 0.72$; $P < 0.05$); however, rates of synthesis [6-12 μmol/(kg·h)] were much lower than in enterally-fed piglets receiving a similar diet.

In enterally-fed piglets, citrulline, but neither ornithine nor proline, addition to the arginine deficient diet was as effective as arginine addition in improving whole-body arginine status. The supplemental ornithine was extensively metabolized to carbon dioxide and proline. The limiting step in the conversion of proline to arginine was

identified as citrulline formation, and this was not affected by arginine intake. Proline conversion to arginine represented 63% and 56% (pooled SE = 5%) of total arginine synthesis in enterally-fed piglets receiving generous and deficient intakes, respectively ($P > 0.05$).

The whole-body rate of nitric oxide synthesis [μmol/(kg·h)] was greater in piglets receiving the generous arginine diet than in those receiving the deficient arginine diet (105 vs. 46; pooled SE = 10) ($P < 0.05$), and this represents one possible way that supplemental arginine may prevent certain neonatal pathologies.

To all those

who have encouraged and supported me

on my journey to find new answers

ACKNOWLEDGEMENTS

I would like to thank Dr. Ronald Ball and Dr. Paul Pencharz for their guidance and supervision during my PhD research and for fostering my interest in studying amino acid metabolism. Hopefully all of your lessons will serve me well for many, many years of research to come. I would also like to thank you both for encouraging me to develop my own research questions throughout my program and to follow them through to completion. Your support has been greatly appreciated. I would also like to acknowledge Drs. Doug Korver and Catherine Field for serving as members of my supervisory committee throughout my PhD program.

A huge thank you must be extended to Dr. Anna Kate Shoveller for all of her support through both my undergraduate and doctoral degrees. Thank you so much for encouraging me into the research world and for teaching me all that there is to know about conducting research in baby piglets. All of your assistance and encouragement will never be forgotten.

I would like to acknowledge all of the other members of the Ball-Pencharz group over the years. Special thanks to Dr. Soenke Moehn for his assistance with everything from experimental design to lab equipment repairs, to Dr. Desmond Pink for all of his help and support during the first years of my program, to Christine Pendlebury and Ryan Samuel for their help with my final trials and for keeping me sane while I was writing my thesis, and to Mahroukh Rafii for all of her work in developing the analysis method for the final study of this thesis.

To the staff of the Swine Research and Technology Centre, Jay Willis, Janes Goller, Dianna Agate, Tamara Staska, and Pamela Wizzard: thank you for making the

SRTC such a wonderful environment to conduct research! I would also like to thank Dr. Richard Uwiera, Dr. Craig Wilkinson, Dr. Nick Nations, Brenda Tchir and Charlane Gorsak for their assistance with the piglet surgeries and necropsies. Thank you to all of the staff and other graduate students of AFNS for your support throughout. A special thanks to Jody Forslund for always having the answers.

And finally, a big thank you to my parents for their support over the many, many years of my post-secondary education and for making an effort at trying to understand what exactly my thesis is about.

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LIST OF ABBREVIATIONS

+ α-KG- α-ketoglutarate supplemented diet (Chapter 6.0)
+a-KG/+Orn- α-ketoglutarate and ornithine supplemented diet (Chapter 6.0)
+Arg- arginine supplemented or generous arginine diet (Chapters 5.0, 7.0)
+Cit- citrulline supplemented diet (Chapter 5.0)
+Orn- ornithine supplemented diet (Chapters 5.0, 6.0)
+Pro- proline supplemented diet (Chapter 5.0)
-Arg- arginine-deficient diet (Chapter 7.0)
ADC- arginine decarboxylase
ADMA- asymetric dimethylarginine
AGAT- arginine:glycine aminotransferase
APE- atom percent excess
ASL- argininosuccinate lyase
ASS- argininosuccinate synthetase
BSA- bovine serum albumin
DDAH- dimethylarginine dimethylaminohydrolase
cNOS- constitutive nitric oxide synthase (eNOS + nNOS)
CPS I- carbamoyl phosphate synthetase I
eNOS- endothelial nitric oxide synthase
iNOS- inducible nitric oxide synthase
LC-MS: liquid chromatography- tandem mass spectrometry
L-NAME- N-omega-nitro-L-arginine methyl ester
NAG- N-acetylglutamate
NAGS- N-acetylglutamate synthase
NCG- N-carbamoylglutamate
NEC- necrotizing enterocolitis
nNOS- neuronal nitric oxide synthase
NOS- nitric oxide synthase
NS- not significant
OAT- ornithine aminotransferase
OKG- ornithine α-ketoglutarate
OTC- ornithine transcarbamoylase
P5C- pyrroline-5-carboxylate
PPHN- persistent pulmonary hypertension of the neonate
$Q_{\text{precursor to product}}$- molar conversion of precursor to product amino acid
SA- specific activity
TPN- total parenteral nutrition

1.0 LITERATURE REVIEW

This literature review will begin with a brief discussion of the challenges associated with the nutrition of neonates and the importance of adequate arginine intake in neonatal piglets and infants. The primary focus of this literature review will be the inter-organ metabolism of arginine and its metabolic precursors, specifically glutamine, glutamate, proline, ornithine and citrulline, as they relate to arginine synthesis. The emphasis will be on neonatal metabolism, although important research findings from mature mammals will also be included. The metabolic uses of arginine, arginine requirements and estimated intakes, and unique interspecies differences in arginine metabolism will also be discussed. Finally, this review will conclude with the discussion of the use of the neonatal piglet as an experimental model for the human neonate.

1.1 Introduction

The optimal nutritional management of premature infants remains a major obstacle in their post-natal care (1). Although breast milk is the preferred diet for infants, premature infants may not be able to tolerate enteral feeding due to a variety of complications associated with their prematurity including, but not limited to, the inability to suckle or swallow, immature gastrointestinal motor development, gastrointestinal surgery, the risk of necrotizing enterocolitis (NEC), and respiratory problems (1-3). Total parenteral nutrition (TPN) is the administration of an elemental diet directly into the vein, and is the nutritional strategy that is commonly used in the infants who are unable to tolerate enteral feedings. TPN may also be used in combination with the enteral administration of small amounts of nutrients to stimulate gastrointestinal development in what is known as minimal enteral feeding [as reviewed by (3)]. Regardless of the form of nutrition used for premature infants, it is of the utmost importance to ensure that the growth and development of the neonate are supported without additional metabolic stress (2); therefore, it is necessary to consider the composition of diets given to premature infants and whether they are optimal for enteral and/or parenteral infusion.

Although nitrogen balance (4-6) and isotopic tracer studies (7) in humans show that endogenous arginine synthesis appears to provide enough arginine to meet the metabolic arginine demands in healthy infants, children and adults, premature or health-compromised infants may suffer adverse effects from inadequate dietary arginine intake. As recently reviewed by Wu et al. (8), preterm infants appear to be particularly at risk for hypoargininemia and this could result in hyperammonemia and increased susceptibility to neonatal pathologies including NEC and persistent pulmonary hypertension of the neonate (PPHN). Regardless of whether enterally (9,10) or parenterally (11) fed, the plasma arginine concentration of premature infants is ~50% of the plasma arginine concentration of full-term, breast-fed infants (12). Low plasma arginine concentrations or whole-body arginine flux have been associated with both NEC (11,13,14) and PPHN (15,16). Furthermore, the administration of supplemental arginine has been shown to improve oxygenation in infants with PPHN (17), prevent the onset of hyperammonemia in both parenterally (18) and enterally-fed preterm neonates (10), and decrease the incidence of NEC in premature infants (13). The protective effect of arginine administration against NEC and PPHN is believed to be due, at least in part, to its role as a nitric oxide precursor (15-17,19). Clearly arginine metabolism in infants, particularly premature infants, is an important aspect of neonatal nutrition.

One of the major obstacles in ensuring adequate arginine intake in neonates is that there is presently no available data with regards to the metabolic arginine requirement in infants. Furthermore, there is a very wide range of arginine contents in the various neonatal diets: breast milk, enteral formulas and TPN solutions, as shown in **Table 1.1**. Similac Special Care (Ross Products Division, Abbott Laboratories), a commonly used pediatric enteral formula in North American hospitals, contains 63 mg/100 mL arginine (13) and 1.66 g/100 mL protein (20); therefore arginine is 3.8% of total amino acids in this diet. Breast milk (3.6% of total amino acids) and enteral formulas (2.9% of total amino acids) contain similar amounts of arginine to the Similac diet (**Table 1.1**). The range of arginine provided in parenteral solutions ranges from 4.7 to 12.2% of total amino acid intake (21), which in all cases is greater than the enteral diets. The amino acid profile of many parenteral solutions is based on oral reference proteins such as egg and human milk protein (2,21), and therefore may not be appropriate for parenterally-fed neonates where intestinal metabolism is bypassed and intestinal atrophy occurs (22). Although the amount of arginine provided in breast milk may be enough for healthy neonates, it may not be enough for premature neonates where the arginine synthetic pathways may not be fully developed (reviewed in 8), as indicated by the previously mentioned neonatal pathologies.

In order to fully understand arginine metabolism and requirements in neonates, additional research is required. However, conducting research in premature human infants is difficult for a number of reasons: 1) they represent a heterogeneous population

Table 1.1 Amino acid composition of parenteral solutions, an enteral formula, maternal milk, and human and porcine tissue

	Aminosyn (Abbott)[1]	Primene (Baxter)[1]	Travasol (Baxter)[1]	Trophamine (McGraw)[1]	Vamin (Pharmacia &Upjohn)[1]	Vaminolact (Pharmacia &Upjohn)[1]	Baby-Semp 2 (Semper AB)[2]	Human milk[3]	Sow milk[3]	Human tissue[4]	Pig tissue[5]
					mg amino acid/g total amino acid						
Alanine	129	79	207	54	43	97	42	40	36	72	72
Arginine	99	84	112	122	47	63	29	36	44	77	69
Aspartate	0	60	0	32	59	63	95	86	78	90	117
Cysteine	0	19	0	1	20	15	15	20	16	NR[6]	NR
Glutamate	0	99	0	50	129	109	202	190	208	130	134
Glycine	129	40	103	36	30	32	18	22	32	118	91
Histidine	30	38	48	48	35	32	24	23	24	26	28
Isoleucine	73	67	60	82	56	55	61	53	40	35	38
Leucine	95	99	73	140	75	108	100	104	89	75	72
Lysine	73	109	58	82	55	86	89	71	79	72	75
Methionine	40	24	40	34	27	20	22	16	22	20	20
Ornithine	0	22	0	0	0	0	0	NR	NR	--	--
Phenylalanine	47	42	56	48	79	42	39	37	43	41	42
Proline	87	30	68	68	116	86	78	95	117	84	60
Serine	42	40	50	38	107	58	55	61	51	44	48
Taurine	0	6	0	2	0	5	3	NR	NR	--	--
Threonine	52	37	42	42	43	55	58	44	37	41	37
Tryptophan	16	20	18	20	14	22	15	NR	NR	NR	NR
Tyrosine	9	9	4	23[7]	7	8	38	46	39	29	32
Valine	81	76	58	78	61	55	65	51	46	47	52

[1] Values for the parenteral formulas calculated by converting the values provided in (21) from % of total amino acids to mg amino acid/g total amino acids.

[2] Baby-Semp 2 is an oral pediatric formula. Values calculated by converting the values provided in (23) from µmol/l of formula to mg amino acid/g total amino acids.

[3] Values provided in (24) in mg amino acid/g total amino acids.

[4] Values provided in (25), which used the mean of the values of 160-180 d, 180-200 d, 200-220 d, 220-240 d, 240-260 d, 260-280 d old fetuses from (26) (in mg/g total amino acids).

[5] Values provided in (25), which used the mean of the values from (27) where the amino acid composition of newborn, weanling, 33-kg and 93-kg pigs was determined (in g/16 g nitrogen), (28) where the composition of 14-, 28- and 56- day old piglets was measured (in mg/g total amino acids), and (29) where the composition of 3- and 9-week old piglets was determined (in g/16 g nitrogen).

[6] NR = not reported

[7] Tyrosine was provided as L-tyrosine (7 mg/g total protein) and N-acetyl-tyrosine (16 mg tyrosine/g total protein).

and may be afflicted with a number of different illnesses, 2) difficulty recruiting experimental subjects leading to small sample sizes, and 3) ethical and practical constraints limit invasive research in human neonates and clinically acceptable research techniques may not be sensitive enough to detect small differences (2,30). For this reason it was necessary to develop and validate an experimental model for amino acid metabolism in neonates. The piglet has been identified as an appropriate model for the parenterally-fed neonate (2,30) and has been extensively used to study amino acid metabolism in enterally-fed neonates (22,31-33), and is a good research model for NEC (19). The composition of sow's milk is very similar to human breast milk and the composition of piglet and human tissues are also highly comparable (**Table 1.1**). The neonatal piglet may be a particularly appropriate model to study neonatal arginine metabolism, because similar to humans, the piglet is also susceptible to hyperammonemia due to dietary arginine deficiency, particularly during parenteral diet administration (34).

Using both the enterally- and parenterally-fed piglet models, several aspects of arginine metabolism in neonates have been elucidated. First, intact first-pass splanchnic metabolism is critical for endogenous arginine synthesis (32-34). First-pass splanchnic metabolism refers to the metabolism of enterally-administered nutrients by the intestine (first-pass intestinal metabolism) and liver (first-pass hepatic metabolism) before they are released into general circulation. When piglets are parenterally-fed, first-pass splanchnic metabolism is bypassed and intestinal atrophy occurs (22), both of which could be potential reasons why parenterally-fed piglets can not endogenously synthesize enough arginine to prevent hyperammonemia when fed an arginine-free diet (34). The portal-drained viscera of enterally-fed piglets releases arginine, equivalent to 137% of arginine intake, to the rest of the body, providing additional evidence that the intestine is an important site of endogenous arginine synthesis (35). Although first-pass intestinal metabolism was identified as a major site of endogenous arginine synthesis (32,33), it only accounted for 40-60% of whole-body arginine synthesis in enterally-fed piglets (33). Therefore, other sites of arginine synthesis must be present in neonatal piglets and these sites need to be determined. Second, proline and not glutamine/glutamate was the major arginine precursor in week-old piglets (33,34); however, there is an upper limit to the ability of proline to act as an arginine precursor, indicating a rate limitation in the proline to arginine metabolic pathway (33). The limitations to endogenous arginine synthesis in neonates also require further investigation.

Not only will a better understanding of arginine metabolism in neonatal piglets have important clinical implications for premature and health-compromised human neonates, but it will also help ensure optimal piglet nutrition. Although arginine requirements are published for 3-5 kg piglets (36), these requirements were extrapolated from larger pigs; therefore, they may not be reflective of developmental and age-specific changes in arginine metabolism. *In vitro* research using isolated enterocytes shows that marked changes in arginine metabolism occur from birth until well after weaning (37-40); therefore, it cannot be assumed that requirements for older, larger piglets can be applied to young piglets. Arginine intake from sow's milk is only about ~40% of the daily estimated arginine use (41), showing that suckling piglets rely heavily on endogenous arginine synthesis to maintain whole-body arginine status. However, even the combination of arginine intake from sow's milk and endogenous arginine synthesis is insufficient to support maximal piglet growth (42), and arginine supplementation may improve rates of piglet growth (42). A better understanding of arginine metabolism in piglets may lead to nutritional strategies for young piglets that could improve growth and lead to greater profitability for swine producers. Therefore using the week-old piglet to study arginine metabolism will produce data that will be relevant for human nutritionists and clinicians as well as swine nutritionists.

1.2 Arginine metabolism

1.2.1 *General overview of arginine synthesis and metabolism*

Arginine is a dibasic amino acid containing four nitrogen atoms, and is also the most abundant nitrogen carrier in the body (43). Endogenous arginine synthesis begins in the mitochondria, and the first nitrogen atom and the five carbon backbone come from the primary dietary precursors for arginine: proline, glutamine and glutamate, which must all be converted to pyrroline-5-carboxylate (P5C). The source of the second nitrogen is from an α-amino acid, such as glutamate, which is transaminated with P5C to form ornithine and an α-ketoacid, such as α-ketoglutarate. The third nitrogen is added when carbomoyl-phosphate is combined with ornithine to form citrulline, which is then transported into the cytosol by the ORNT1 transporter, and the final nitrogen comes from aspartate in the formation of argininosuccinate, which subsequently releases a fumarate molecule to form arginine. The urea cycle involves many of the same enzymatic steps as endogenous arginine synthesis including the mitochondrial synthesis of carbamoyl phosphate from ammonia and bicarbonate, the reaction of carbamoyl phosphate and ornithine to form citrulline, the transport of citrulline into the cytosol, followed by argininosuccinate and subsequently arginine formation. Arginine is then hydrolyzed to form urea and ornithine. The ornithine is then transported back to the mitochondria where it can be used in another rotation of the urea cycle. The metabolic pathways involved in arginine synthesis and metabolism have been previously reviewed by others (41,44,45), and are depicted in **Figure 1.1** and explained in **Table 1.2**, which also lists the enzymes involved in catalyzing each of these reactions.

Figure 1.1 Pathways of arginine metabolism

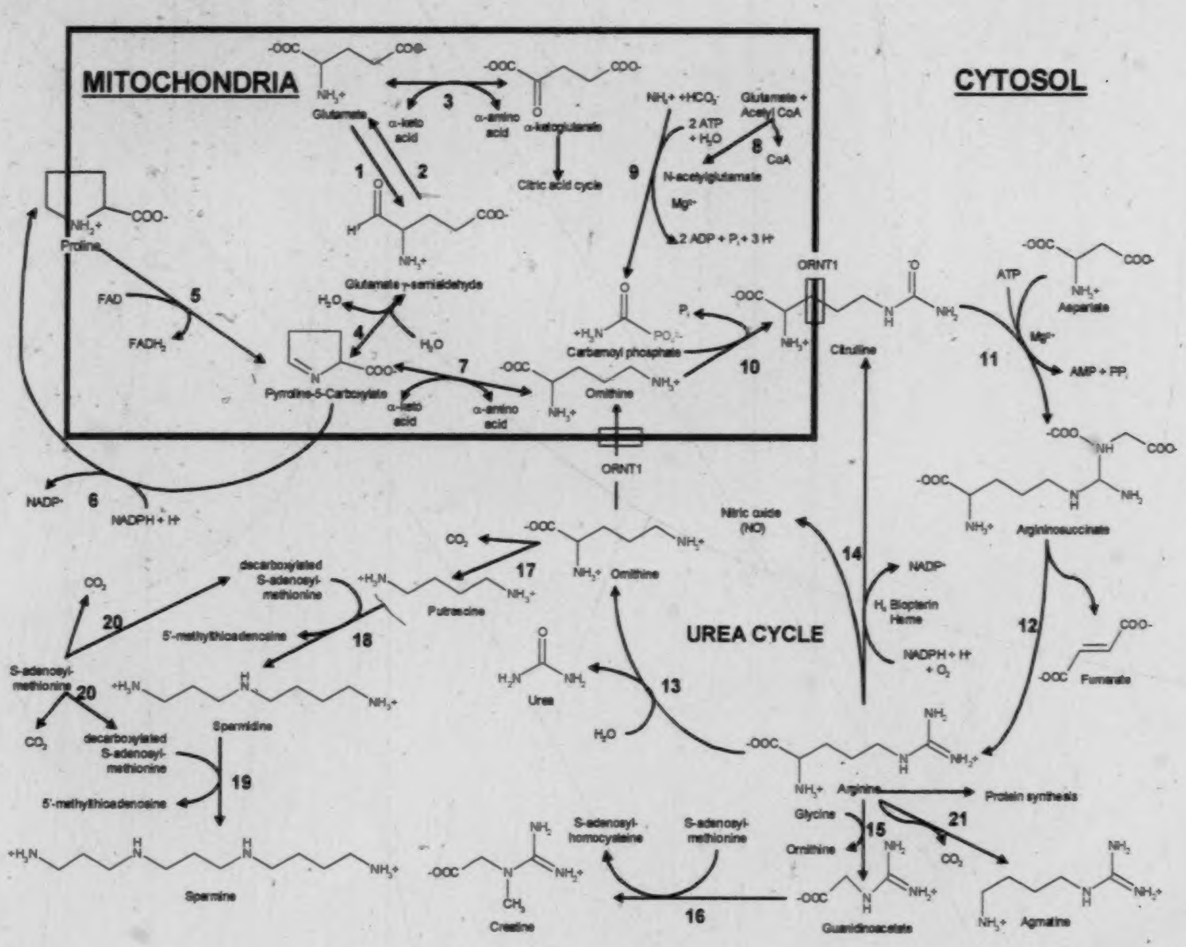
MITOCHONDRIA
CYTOSOL
UREA CYCLE
Proline
FAD
FADH2
5
Glutamate
α-keto acid
α-amino acid
3
α-ketoglutarate
Citric acid cycle
1
2
Glutamate γ-semialdehyde
H2O
4
Pyrroline-5-Carboxylate
7
Ornithine
NH4+ + HCO3-
2 ATP + H2O
9
Mg2+
2 ADP + Pi + 3 H+
Glutamate + Acetyl CoA
8
CoA
N-acetylglutamate
Carbamoyl phosphate
Pi
10
ORNT1
Citrulline
ATP
Aspartate
Mg2+
AMP + PPi
11
Argininosuccinate
12
Fumarate
Arginine
Protein synthesis
14
NADP+
H4 Biopterin Heme
NADPH + H+ + O2
Nitric oxide (NO)
13
Urea
Ornithine
Glycine
15
Guanidinoacetate
S-adenosyl-methionine
S-adenosyl-homocysteine
16
Creatine
21
CO2
Agmatine
17
Putrescine
decarboxylated S-adenosyl-methionine
18
5'-methylthioadenosine
Spermidine
19
Spermine
20
6
NADP+
NADPH + H+

Table 1.2 Enzymes of the metabolic pathways illustrated in Figure 1.1

Number	Enzyme	EC number
1	Pyrroline-5-carboxylate synthase (P5C synthase)	Not assigned
2	Pyrroline-5-carboxylate dehydrogenase (P5C dehydrogenase)	1.5.1.12
3	Aminotransferase enzyme (ie. alanine aminotransferase)	2.6.1.12
4	Spontaneous reaction	N/A
5	Proline oxidase	1.5.99.8
6	Pyrroline-5-carboxylate reductase (P5C reductase)	1.5.1.2
7	Ornithine aminotransferase (OAT)	2.6.1.13
8	N-acetylglutamate synthase (NAGS)	2.3.1.1
9	Carbamoyl phosphate synthetase I (CPS I)	6.3.4.16
10	Ornithine transcarbamoylase (OTC)	2.1.3.3
11	Argininosuccinate synthetase (ASS)	6.3.4.5
12	Argininosuccinate lyase (ASL)	4.3.2.1
13	Arginase (also a mitochondrial enzyme in some tissues)	3.5.3.1
14	Nitric oxide synthase (NOS)	1.14.13.39
15	Arginine:glycine aminotransferase (AGAT) (mitochondrial enzyme)	2.1.4.1
16	Guanidinoacetate N-methyltransferase	2.1.1.2
17	Ornithine decarboxylase (ODC)	4.1.1.17
18	Spermidine synthase	2.5.1.16
19	Spermine synthase	2.5.1.22
20	S-adenosylmethionine decarboxylase	4.1.1.50
21	Arginine decarboxylase (ADC) (mitochondrial enzyme)	4.1.1.19

Not only is arginine needed for protein synthesis, but other important metabolic roles include as a precursor for nitric oxide, creatine, polyamine and agmatine synthesis (reviewed in 41,44,45), as an activator of N-acetylglutamate synthase (NAGS), as a secretagogue for hormones such as insulin, glucagon, prolactin, growth hormone and steroids (reviewed in 46), and as a regulator of the immune response (reviewed in 47). The primary route of arginine degradation is through its hydrolysis to urea and ornithine, with the ornithine subsequently being converted back to P5C and then to glutamate, which enters the citric acid cycle via α-ketoglutarate. These pathways will be covered in more detail in the sections that follow and are also shown in **Figure 1.1**. The role of arginine in the immune system and as a secretagogue for the hormones listed above will not be covered in this literature review and the reader is directed to previously published review articles for further information (46,47).

1.2.2 *Arginine transport*

The transport of arginine across plasma membranes has been extensively reviewed elsewhere (48,49), and the key features of the various arginine transporters are summarized in **Table 1.3**; therefore, only the key features will be mentioned in-text. There are four different transport systems responsible for the influx and efflux of arginine, and other cationic amino acids such as lysine, histidine and ornithine, in the various cell types of the body: the y^+ system, a fairly ubiquitous system that has four different protein isoforms; the y^+L system, a heterodimeric system that also transports the large neutral amino acids; the $b^{0,+}$, another heterodimeric system that transports neutral

Table 1.3 Key features of the plasma membrane transporters for arginine[1]

Transport system	Protein[2]	Na^+-dependent?	Apparent K_m for arginine (mmol/L)[3]	Tissues expressing this transport system
y^+	CAT-1	No	0.10 – 0.16	Almost all tissues except the liver; expressed on the basolateral membrane of small intestinal enterocytes and kidney cells
	CAT-2A	No	3.40 – 3.90	Primarily liver, lower expression in the pancreas, skeletal muscle, cardiac and vascular smooth muscle cells
	CAT-2B	No	0.25 – 0.70	Cells with iNOS activity; may be co-induced with iNOS
	CAT-3	No	0.20 – 0.50	Widely expressed during embryonic development; in adult animals in brain, neurons, thymus, testis and mammary glands
y^+L[4]	y+LAT1 + 4F2hc	No	0.34	Basolateral membrane of small intestinal enterocytes and kidney cells, other non-polarized cells
	y+LAT2 + 4F2hc	No	0.12 – 0.14	Basolateral membrane of colonocytes, other non-polarized cells
$b^{0,+}$[5]	$b^{0,+}$AT + rBAT	No	0.08 – 0.20	Apical membrane of small intestinal enterocytes and kidney cells
$B^{0,+}$[6]	$ATB^{0,+}$	Yes	0.10 – 0.15	Most abundant in the lung and salivary glands; other expression on the apical membrane of colonocytes, mammary gland, pituitary gland, stomach

[1] Data from this table adapted from (48). [2] Protein names are for the human isoforms. [3] K_m of arginine for the human isoforms. [4] In addition to the cationic amino acids, other amino acids transported by y^+L include the aromatic and branched chain amino acids. [5] In addition to the cationic amino acids, other amino acids transported $b^{0,+}$ include neutral amino acids and cystine. [6] In addition to the cationic amino acids, other amino acids transported by $B^{0,+}$ include neutral amino acids.

amino acids and cystine; and $B^{0,+}$, a Na^+ and Cl^--dependent system that also transports the neutral amino acids (48).

The y^+ system for amino acid transport was the first system described in mammals for cationic amino acids (50), and further research has revealed that there are at least 3 different proteins involved in this system in a variety of tissue types: CAT -1, -2 and -3, with CAT-2 having two different splice variants, CAT-2A and CAT-2B. Each different protein has unique tissue expression and kinetic features (**Table 1.3**) (51,52). Of particular note is CAT-1, which is the most researched of the y^+ proteins, and is expressed in nearly all tissue types, with the notable exception being hepatocytes (53). In cell culture, the mRNA of the CAT-1 gene is increased in abundance when amino acids are removed from the culture medium, and this was due to the increased stability of the mRNA, which resulted in an increase in CAT-1 protein translation (54). The implication of this is may be that *in vivo* in times of amino acid, specifically arginine, deficiency, cells expressing the CAT-1 gene may be able to sustain arginine transport into the cytoplasm, despite low circulating concentrations. The primary form of y^+ transport protein in the liver is the CAT-2A isoform which has a much lower affinity for arginine (**Table 1.3**; ~3.5 mmol/L for CAT-2A versus 0.10 – 0.70 mmol/L for other CAT isoforms) than the other isoforms. The role of CAT-2A in the liver has been suggested as a way to clear high plasma arginine concentrations from the blood during times of catabolic stress (48). Because the expression of both CAT-2B and inducible nitric oxide synthase (iNOS) are increased by cytokines and lipopolysaccharide in certain cell-types such as macrophages (55), this isoform of the arginine transporter may be particularly important for the transport of arginine for nitric oxide synthesis in times of injury or infection. iNOS activity appears to be dependent on extracellular arginine (56), and therefore by having an arginine transporter associated with iNOS, this ensures a high availability of substrate for nitric oxide synthesis.

In most cell types, the influx and efflux of arginine appears to be a cooperative effort between different cationic amino acid transport systems. In the small intestinal and renal apical membrane cells, arginine enters the cells primarily via $b^{0,+}$ system and leaves via either the y^+ or y^+L systems of the basolateral membrane (48,57). During arginine-uptake by the $b^{0,+}$ system, an equimolar amount of neutral amino acids is transported out of the cell, and during arginine release by the y^+L system, neutral amino acids are transported into the cell (48). Transport by the y^+ systems involves the exchange of one cationic amino acid for another (48). In non-polarized cells, the y^+ system is involved in both the influx and efflux of arginine, while the y^+L system is involved primarily in arginine efflux (48).

In terms of arginine transport throughout the body via the circulatory system, arginine in the blood does not equilibrate between the plasma and the blood cells (58), therefore plasma must be used as the sampling pool in isotope kinetics studies. This was illustrated by the fact that when radioactive arginine was infused in mice, the specific activity of arginine and arginine metabolites (ie. citrulline, ornithine) was much greater in plasma than in blood cells (58). The lack of equilibrium between the plasma and blood cell arginine compartments was attributed to the fact that, compared to the system L transport protein, the system y^+ transport protein was less abundant in human erythrocytes (59). System L is responsible for the cellular transport of the large neutral amino acids such as phenylalanine, leucine and valine, three amino acids which do equilibrate well between the plasma and blood cell compartments (58); therefore based on these observations it appears that the ability of amino acids to equilibrate between the plasma and blood cell pools is very dependent on the ability to transport these amino acids into the erythrocytes.

1.2.3 *Endogenous arginine synthesis in adult and neonatal mammals*

The importance of endogenous arginine synthesis was first described over seventy-five years ago in rats, when it was discovered that rats accreted 2-3 times more arginine in their tissues than was provided in the diet (60). Research since then has focussed on the sites of this endogenous synthesis. In adult mammals, the intestinal-renal axis has been well-established as the site of endogenous arginine synthesis. In a series of perfusion and mass balance studies, Windmueller and Spaeth (61-64) demonstrated that there was extensive small intestinal metabolism of glutamine and glutamate (62,63) and the release of citrulline to the rest of the body (61,62). The intestine was identified as the major site of synthesis for circulating citrulline (61).

The importance of the intestinal citrulline synthesis has been demonstrated in several studies. In the rat by the specific inhibition of intestinal ornithine transcarbamoylase (OTC) activity using an infusion of a glycyl-glycyl derivative of δ-N-(phophonacetyl)-L-ornithine, where the rats lost weight or gained much slower than when OTC activity was present (65). The growth retardation could only be completely reversed by the addition of citrulline to the diet (65). That the intestine is critical for endogenous arginine synthesis has been demonstrated in humans with low intestinal mass due to diseases such as short bowel syndrome. Small intestinal length ($r^2 = 0.57 – 0.65$), and surface area ($r^2 = 0.69$) are strongly related to plasma citrulline concentrations in adults with short bowel syndrome (66). Similarly, plasma citrulline concentrations are an effective diagnostic tool in predicting small intestinal mass ($r^2 = 0.66$) in adults with villus atrophy (67).

The intestine of weaned rats and pigs is not a site of net arginine release. In rats, although both [^{14}C]glutamine and glutamate were extensively metabolized to other products, including citrulline, labelled arginine was not detected (62). The incubation of weaned pig enterocytes with [^{14}C]proline did not result in any detectable radioactivity in arginine (40), although [^{14}C]citrulline was formed, providing further confirmation that the intestine of weaned pigs is a site of citrulline, but not arginine, release.

The ability of the kidney to convert citrulline to arginine in adult mammals is not a recent discovery, with *in vitro* studies in kidney slices demonstrating arginine formation from citrulline (68,69) in the 1940s and 1950s. The fact that the kidney is the major biosynthetic organ for circulating arginine in adult rats was identified 20-30 years later, using ligation of the kidneys and/or liver and demonstrating that the incorporation of radioactive citrulline into the protein of other tissues, was virtually abolished when the kidney, but not the liver, was ligated (70). The study by Windmueller and Spaeth revealed that 83% of the citrulline released by the intestine was taken up by the kidneys, and 75% of this citrulline was converted to arginine and released to the rest of the body (61). A later study, using similar arterio-venous difference methodology to the previous study (61), in addition to the infusion of citrulline to create a wider range of renal citrulline uptake rates, showed that there was a strong ($r^2 = 0.85$) linear relationship between the arterio-venous differences of citrulline and arginine across the kidney, with a slope of ~ 1 (71). Therefore, the renal output of arginine was dependent on circulating citrulline concentrations (71). The cortical region of the kidney, more specifically the proximal convoluted tubule, is the primary site of citrulline conversion to arginine in adult rats (72,73).

In the neonatal mammal, however, the small intestine is capable of the entire *de novo* synthesis of arginine. The initial evidence for intestinal arginine synthesis was from an *in vitro* study using the intestine of mice from birth through to adulthood that found that the formation of arginine from either ammonia + CO_2 or from citrulline, and the incorporation of the newly formed arginine into intestinal protein, was much higher in newborn versus adult mice (74). This corresponded to an increase in intestinal arginase activity with age and a decrease in intestinal ASL activity (74). The potential ability of the newborn mouse intestine to synthesize arginine is further supported by the fact that the ORNT1 transporter mRNA was detected in the small intestine of fetal and newborn mice, and the mRNA abundance increased rapidly in the first two weeks of life (75).

The intestinal synthesis of arginine has been extensively studied *in vitro* in piglets from birth through weaning. The suckling piglet intestine has measurable activity of all of the enzymes involved in the arginine synthetic pathway (**Figure 1.1**, **Table 1.2**) (37-39,76), and the activity levels of these enzymes from birth through until after weaning are presented in **Table 1.4**. Briefly, from birth until weaning there was a decrease in proline oxidase, P5C synthase, CPS I, ASS and ASL activities (**Table 1.4**) (37-39), intestinal OAT activities increased (38,39) and arginase activity increased slightly, although it was still virtually negligible (77). In isolated enterocytes

Table 1.4 Activities of enzymes involved in arginine metabolism in the neonatal piglet

	Piglet age							
	0 d[1,2]	2 d[2,3]	4 d[3]	7 d[3]	14 d[3]	21 d[3]	29 d[4]	58 d[4]
Intestine								
Proline oxidase[5] [nmol/(mg protein·min)]	28.2	25.1		6.4	9.7	13.6	14.5	15.2
P5C synthase[6,7] [pmol /(mg protein·min)]	1108	367		370	12	17	272	240
Ornithine aminotransferase[6,7] [nmol ornithine/(mg protein·min)]	114	306		335	333	123	65	28
Ornithine aminotransferase[5] [nmol P5C/(mg protein·min)]	353	672		625	605	372	259	236
Carbamoyl phosphate synthetase I[6,7] [nmol/(mg protein·min)]	3.26	2.11		2.04	2.20	2.31	1.08	1.18
Ornithine transcarbamoylase[6,7] [nmol/(mg protein·min)]	133	140		147	129	97	60	26
Argininosuccinate synthetase[8] [nmol/(mg protein·min)]	1.61	1.52		1.58	0.38	0.43	1.81	1.72
Argininosuccinate lyase[8] [nmol/(mg protein·min)]	19.3	3.14		3.28	3.02	2.86	2.91	3.08
Arginase[8] [nmol/(mg protein·min)]	0.019	0.021		0.020	0.023	0.047	2.51	2.71
Nitric oxide synthase[9] [pmol/(mg protein·min)]	53.7		28.3	17.5	7.3	11.5	48.6	51.7
Ornithine decarboxylase[9] [pmol/(mg protein·min)]	1.06		0.27	0.24	0.53	0.61	1.52	1.48
P5C reductase[9] [nmol/(mg protein·min)]	61.7		40.1	35.7	38.2	34.7	55.8	59.3
P5C dehydrogenase[9] [nmol/(mg protein·min)]	0.33		0.36	0.40	1.42	1.61	1.70	1.39
Kidney								
Argininosuccinate synthetase[6] [nmol/(mg protein·min)]	8.89	9.08		8.84	9.32	7.26	5.76	5.73
Argininosuccinate lyase[6] [nmol /(mg protein·min)]	1.57	2.98		3.47	4.72	6.45	7.88	8.05

[1] Samples from 0 d old piglets were taken prior to suckling. In all studies piglets were weaned at 21 d of age. [2] 2 d old piglets had very high intestinal protein content (37), which was believed to be due to the uptake of colostral immunoglobulins; therefore, the intestinal activity of enzymes that are expressed on a per mg protein basis may underestimated. [3] Tissue samples were taken from suckling piglets. [4] Tissue samples were from weaned piglets. [5] Data from (39). [6] Data from (37). [7] Data from (38). [8] Data from (77). [9] Data from (78).

from suckling piglets there was a significant conversion of radioactive label from proline to arginine (39), a net production of arginine from citrulline (37), and the net conversion of glutamine to citrulline (37). Therefore, in young piglets, isolated small intestinal cells are capable of converting precursor amino acids, proline and glutamine, to citrulline and subsequently arginine.

A series of studies in the neonatal piglet model have investigated sites of arginine synthesis. Parenterally-fed piglets, with small intestinal atrophy (22), become hyperammonemic more rapidly than enterally-fed piglets when given an arginine-free diet, even when this diet contained the arginine precursors glutamate and proline (34). This demonstrated that intact first-pass splanchnic metabolism was critical for endogenous arginine synthesis. However, from these observations it was not possible to conclude whether the inability of parenterally-fed piglets to synthesize arginine (34) was due to the bypass of either first-pass intestinal or hepatic metabolism, or due to decreased intestinal blood flow (79) and intestinal atrophy (22) resulting from the parenteral feeding. It was later revealed using the infusion of radio-isotopes that first-pass intestinal metabolism was critical for the conversion of proline to ornithine (32). However, first-pass intestinal metabolism only accounted for 40-60% of whole-body arginine synthesis in week-old neonatal piglets, and therefore other sites of arginine synthesis must be present (33). It is possible that it is the intestinal metabolism of arterial precursors that accounted for the remaining portion of arginine synthesis; however, this was not investigated in the previous study (33).

There appears to be species specific differences in the effects of age on the distribution of ASL activity between the kidney and jejunum. In both mice and piglets, the intestinal activity of ASL, on a nmol/(mg protein·min) basis, is several fold greater than the renal activity at birth (37,74) (**Table 1.4**). In mice, by 12 days of age, the renal activity is several-fold higher than the intestinal ASL activity, and this continues through adulthood (74), this was due to decreases in intestinal ASL activity and increases in renal activity (74). Piglets, on the other hand, have a steady intestinal ASL expression from 2 – 58 d of age [range of 2.86 – 3.28 nmol/(mg protein·min)], with weaning occurring at 21 d of age, and kidney activity increases gradually with age (37). The renal ASS activity in piglets was much higher than the intestinal activity in all ages of piglets studied (37). The results from these *in vitro* enzyme studies suggest that it is possible that the kidney may be a quantitatively more important site of arginine synthesis in the neonatal piglet than in neonatal mice. There has been no *in vitro* or *in vivo* research in neonates of any species examining the possible role of the kidney in endogenous arginine synthesis. The *in vivo* isotope data in piglets (33) also do not eliminate the possibility that the neonatal piglet kidney may be involved in a portion of the arginine synthetic pathway, as 40-60% of arginine synthesis did not occur during first-pass intestinal metabolism. The possible involvement of the kidney would likely be similar in neonatal piglets as in adults, specifically the conversion of citrulline to arginine, because the piglet kidney lacks OTC activity (80). However, the proline oxidase activity in the piglet kidney was similar or greater than the intestinal activity in 0 – 7 d old piglets (76), and continued to increase following weaning, and therefore, the role of the neonatal piglet kidney in endogenous arginine synthesis can certainly not be discounted.

Regardless of whether or not the neonatal piglet kidney is involved in endogenous arginine synthesis, there are vast changes in intestinal arginine synthesis that occur with weaning that shift endogenous arginine synthesis from primarily an intestinal function to primarily a renal function. Most notable is the exponential increase in intestinal arginase activity (77,81), which is what is believed to be responsible for the shift from intestinal to intestinal-renal arginine synthesis. The induction of intestinal arginase is most likely due to the cortisol surge that accompanies weaning (82). In the *in vitro* enzyme studies summarized in **Table 1.4**, piglets were weaned at 21 days of age; however, if piglets remained with the sow after 21 days of age, and received daily intramuscular infusions of either sesame oil (vehicle), cortisol, RU-486 (a blocker of the glucocorticoid receptors) or cortisol + RU-486, at 29 days of age piglets in the cortisol group had intestinal arginase activity that was ~6-fold greater than piglets in the other groups (82). In a related study, if 21 d old piglets were given an injection of RU-486 immediately before and 1 and 3 d after weaning, at 29 d of age the intestinal arginase activity of the weaned + RU-486 piglets was 2-fold greater [0.75 nmol/(mg protein·min)] than in piglets that continued to suckle until 29 d of age [0.26 nmol/(mg protein·min)], but was only 27% of the intestinal activity of piglets that were weaned at 21 d of age and not given RU-486 [2.78 nmol/(mg protein·min)] (83). Therefore, the transition from intestinal to intestinal-renal arginine synthesis in piglets is triggered by the cortisol-surge associated with weaning. In a similar manner to arginase, the intestinal activities of P5C synthase and ASL were also induced by cortisol in weaned piglets (82,83).

In adult humans and rats, the rate of endogenous arginine synthesis by the kidney was not affected by dietary arginine intake. In healthy, adult males receiving either an arginine-free or arginine-rich diet, the molar rate of conversion of citrulline to arginine, which is a measure of renal arginine synthesis, was not different between groups [~ 5.5

μmol/(kg·h)] (7). The rate of arginine release from the kidney of adult rats, as determined by an arterio-venous difference study, was not different in rats receiving diets containing 0, 0.5 or 2% arginine [~ 25 μmol/(kg·h)] (84). In week-old enterally-fed piglets, however, the whole-body rate of arginine synthesis was 2 times greater in piglets receiving a deficient [162 μmol/(kg·h)] versus generous [85 μmol/(kg·h)] intake of arginine, but similar to renal arginine synthesis in adult mammals, there was no effect of dietary arginine intake on first-pass intestinal arginine synthesis [~ 60 μmol/(kg·h)] (33). The tissue sites responsible for the increased endogenous arginine synthesis in piglets receiving an arginine-deficient diet have not been identified. Therefore, not only are the rates of arginine synthesis greater in neonatal mammals than in adult mammals, but there are also differences in the dietary regulation of arginine synthesis.

1.2.4 *Contributions of different arginine precursors to endogenous arginine synthesis*

1.2.4.1 Glutamine and glutamate

In vivo research in neonatal piglets showed that glutamate, administered either enterally or parenterally, was not an arginine precursor (33,34,85). Administration of a glutamate containing arginine-free diet either parenterally or enterally could not prevent hyperammonemia (34). Using radio-labelled glutamate, in enterally-fed neonatal piglets, to quantify the contribution of glutamate to whole-body arginine synthesis, and the effect of dietary arginine intake on this contribution, Wilkinson et al (33) could not measure any transfer of label from glutamate to arginine and concluded that glutamate did not significantly contribute to whole-body arginine synthesis, even during times of very low arginine intake.

The most likely reason why dietary glutamine and glutamate are poor precursors for the 5-carbon backbone of arginine is because they are extensively catabolized in the intestine. In the rat intestine, of the glutamine and glutamate absorbed by the jejunum, 56-64% of glutamine and glutamate were oxidized to CO_2 (62). In 2 - 4-wk old milk-replacer-fed piglets, 93 - 95% of the enterally-administered glutamate was extracted by the portal-drained viscera (35,86). 52% of the enteral glutamate was oxidized to CO_2, representing 36% of the total CO_2 produced by the portal-drained viscera (87). Arterially extracted glutamine was also extensively oxidized (70% of the dose) by the portal drained viscera (87). Studies in humans revealed that 96 and 64% of enterally administered glutamate and glutamine, respectively, were extracted during first-pass intestinal metabolism, with oxidation to CO_2 accounting for 78% of the extracted glutamate and 83% of the extracted glutamine (88,89).

In piglets from birth to 58 d of age, the intestinal activity of P5C synthase, the enzyme necessary for glutamine/glutamate use for endogenous arginine synthesis was much lower than proline oxidase activity (37,38) (**Table 1.4**), which could also explain why glutamine and glutamate were not major arginine precursors *in vivo*.

The *in vivo* finding that glutamate is not an arginine precursor, seems to be in contrast to the *in vitro* finding that glutamine addition to the enterocyte incubation medium increases arginine release (37,39). One possible explanation for this paradox is that, although not major sources of the carbon arginine backbone, glutamine and glutamate could be the sources of the nitrogen required for the formation of ornithine, carbamoyl phosphate and aspartate. Therefore, adding glutamine to the incubation medium increases nitrogen available for the formation of these intermediates and therefore would increase arginine formation (37,39).

Glutamine and glutamate may be quantitatively more important precursors for the carbon backbone of arginine in weaned versus newborn mammals because P5C synthase activity is induced by the increase in cortisol at weaning (82,83). This has not been measured *in vivo* in a whole-body model; however, the intestinal metabolism of [U-^{14}C]glutamine and [U-^{14}C]glutamate still produces relatively low amounts of [^{14}C]citrulline (3-6% of the total metabolic products), compared to other metabolic products (62), further supporting the position that there is little physiological importance of glutamine and glutamate as arginine precursors.

1.2.4.2 Proline

In enterally-fed neonatal piglets, proline is the main arginine precursor (33,34,85). Proline addition to an arginine-free diet given enterally was able to prevent hyperammonemia for the eight-hour duration of the study (34). Follow-up research showed that the radioactive label from proline was transferred to ornithine, citrulline and finally arginine (32,33). Because no arginine synthesis from glutamate could be detected, for neonatal piglets arginine synthesis was determined to be equivalent to the molar conversion of proline to arginine (33). Sow's milk and human milk are both rich in proline (**Table 1.1**) (24,25,90), and so neonates have a readily available supply proline for use in arginine synthesis. The relative importance of proline as an arginine precursor has not been investigated in any other species.

In spite of proline being the primary dietary precursor for endogenous arginine synthesis, there was an upper limit in its use for arginine synthesis. Piglets receiving an arginine-deficient diet, despite having a higher rate of endogenous arginine synthesis than piglets receiving a generous arginine diet, still displayed symptoms of arginine deficiency, including having a lower arginine flux, lower plasma arginine concentrations, and elevated plasma ammonia and urea concentrations (33). The conversion of proline to arginine in enterocytes was not very efficient, with only ~5 – 7% of [^{14}C]proline-derived P5C being converted to [^{14}C]arginine in 2-wk old piglet enterocytes (91). The limitations in the metabolic pathway between proline and arginine require elucidation.

1.2.4.3 Ornithine

Although the addition of ornithine to an arginine-free or arginine-deficient diet was effective at reducing plasma ammonia concentrations in cats (92), dogs (93) and rats (94), it was not a precursor for circulating arginine in cats (92), dogs (93), pigs (95) or rats (94). Dietary ornithine supplementation did not increase portal vein plasma citrulline concentrations, relative to an arginine-deficient diet, in rats (94); therefore, it did not appear to be utilized as a citrulline precursor during intestinal metabolism.

Other possible metabolic fates of supplemental ornithine include conversion to other metabolites, such as proline or polyamines, oxidation to CO_2, or uptake by the liver to replenish urea cycle intermediates. Ornithine can be taken up by the liver, as demonstrated by perfusion studies in rats (96). In addition, ornithine addition to the perfusion solution increases urea production by 6-fold and reduces hepatic ornithine production, therefore it is possible that the major metabolic fate of supplemental dietary ornithine is uptake by the liver and use in urea cycle function (96). In neonatal piglets receiving a diet devoid of arginine, 25% of an enterally-administered [1-^{14}C]ornithine was converted to CO_2, whereas 36% of the ornithine isotope was oxidized when the isotope was infused into the portal vein (32). The fact that ornithine oxidation was higher when the isotope was exposed to hepatic metabolism before either intestinal or peripheral metabolism demonstrates that, *in vivo* the liver is capable of ornithine oxidation. Furthermore, the liver contains the enzymes that are necessary for ornithine oxidation (97). The preferential oxidation of ornithine to CO_2 is in agreement with the observation that humans with an inborn error affecting the OAT gene have an accumulation in ornithine and do not experience hyperammonemia (98), suggesting that under normal conditions *in vivo* the metabolism of ornithine to P5C via OAT is more favoured than the conversion of P5C to ornithine. This is reflected in porcine enterocytes where the activity of OAT in the direction of ornithine formation was only 12 - 54 % of the activity in the direction of P5C formation in all ages of piglets studied (37,39).

1.2.4.3.1 Ornithine α-ketoglutarate

Ornithine α-ketoglutarate (OKG) is an ionic salt that contains 2 mols of ornithine and 1 mol of α-ketoglutarate. OKG has been used clinically as a means to detoxify ammonia in end stage liver disease patients, where it was successful in reducing plasma ammonia concentrations, presumably by promoting urea cycle function (99). Further research found that OKG addition to enteral and parenteral feeding solutions resulted in improved nitrogen balance in burn (100), and post-surgical patients (101), better growth rates in parenterally-fed children (102), improved wound healing following thermal injury (100), improved intestinal structure and function following resection (103,104) and altered amino acid metabolism (105). OKG is a secretagogue of growth hormone, IGF-1 and insulin (reviewed in 106), and this may partially explain its beneficial effects on growth and nitrogen balance. However, these effects of OKG were not duplicated by the addition of either ornithine-HCl or an α-ketoglutarate salt alone (105,107), and so it was the combination of these two molecules that were required for the observed effects of OKG, although the mechanism behind these functions of OKG has not been elucidated. Of particular note, is that OKG administration in fasted male subjects increased plasma arginine concentrations, and this could not be duplicated by the administration of ornithine-HCl or calcium α-ketoglutarate alone (105). Cynober (105,106) proposed that because ornithine and α-ketoglutarate are metabolically related by a series of reversible reactions (see **Figure 1.1**, **Table 1.2**), the simultaneous addition of the substrates on either end of this pathway (ornithine and α-ketoglutarate) may allow ornithine to be directed towards metabolic fates such as arginine, proline and polyamine synthesis, instead of catabolism through the citric acid cycle. Unfortunately this mechanism has not been conclusively verified in mammals of any age.

1.2.4.4 Citrulline

In a variety of mammalian species such as cats (92), dogs (93), pigs (95) and rats (94) citrulline can spare the dietary requirement for arginine for growth and urea cycle function. However, citrulline effectiveness as an arginine precursor has not been investigated in newborn mammals.

Citrulline administration may be advantageous over ornithine supplementation in weaned and adult mammals, because unlike ornithine (96) it is not extensively metabolized by the liver (61) or the intestine (94), enabling it to reach the kidney for conversion to arginine. Supplemental arginine was also much more readily extracted by the liver for metabolism (94,96) than supplemental citrulline (94). In addition, in adult mammals, plasma citrulline concentration was the determinant of renal arginine output (71). These observations together suggest that citrulline supplementation may be more effective than arginine at improving whole-body arginine status, as arginine formation is encouraged. Indeed, in rats with an 80% intestinal resection, citrulline supplementation increased plasma arginine concentrations to a greater extent than arginine supplementation at an equimolar rate (108). Citrulline supplementation of the rats with the intestinal resection also resulted in higher nitrogen retention compared to arginine supplementation (108).

1.2.5 *Oxidative catabolism of arginine*

The oxidation of arginine to CO_2 can occur via one of three pathways: the conversion of arginine to ornithine, via arginase, and subsequently to putrescine via ODC, the conversion of arginine to agmatine via ADC, or conversion of the ornithine to glutamate which can subsequently enter the citric acid cycle as α-ketoglutarate (**Figure 1.1, Table 1.2**). The use of arginine for polyamine and agmatine synthesis will be discussed in greater detail in sections 1.2.7.5 and 1.2.7.6; however, from piglet enterocyte data, in all ages of piglets studied the activity of ADC was completely absent and CO_2 production from [U-^{14}C]ornithine virtually eliminated when an exogenous inhibitor of

OAT, gabaculline, was added to the cell incubation medium (78). Therefore, at least in the piglet enterocyte, the main metabolic pathway for arginine oxidation appears to be via the citric acid cycle (78). The intestinal activities of the various enzymes involved in arginine catabolism, and the effect of age on these activities, are presented in **Table 1.4**.

The isoform of arginase that converts arginine to urea in the urea cycle, arginase I, is a cytosolic enzyme that is present in the periportal region of the liver (109). The use of arginine for supporting urea cycle function will be discussed in section 1.2.7.1. The isoform of arginase that is believed to be responsible largely for the oxidative catabolism of arginine, arginase II, is a mitochondrial enzyme, and is present in a variety of mammalian tissues including the brain, small intestine, kidney, skeletal muscle, liver, lung, mammary gland and spleen (109,110). The tissues having the greatest arginase II activity are the small intestine, kidney and lactating mammary gland (110). Mitochondrial arginase II is co-localized with mitochondrial OAT in the kidney (72), intestinal cells also have OAT activity (37,39). In the cortical kidney cells, which are the major site of renal arginine synthesis, there is relatively low arginase II and OAT expression, showing that arginine synthesis and catabolism occur at different sites in the kidney (72). In the rat liver, OAT is located only in the perivenous hepatocytes (111), and these cells must also have arginase II activity as arginine can be catabolized to CO_2 in these cells, as demonstrated by O'Sullivan et al (97) and Nissim et al (112) using retrograde and antegrade liver perfusions. The overall consequence of these research findings is that arginine degraded via arginase II is likely arginine designated for oxidative catabolism, whereas arginine converted to urea by arginase I in the liver is for use in urea cycle function, and these functions occur in different cell types.

No studies could be found in either humans or piglets, of any age, that directly examined the effects of arginine intake on arginine oxidation to CO_2. However, one study in rabbits showed that $^{14}CO_2$ production from [^{14}C]arginine was lower in rabbits receiving deficient intakes of arginine, than in those receiving adequate or excess arginine intake (113). After the arginine requirement was met, there appeared to be an increase in arginine oxidation with arginine intake, and plasma arginine concentrations remained unchanged (113), suggesting that one way to control plasma and tissue arginine concentrations is through arginine oxidation. In humans, ornithine oxidation has been used as a measure of arginine oxidation. The rate of ornithine oxidation was 8, 14-27 and 63 μmol/(kg·h) in males receiving arginine-free, arginine-adequate and arginine-rich diets (114,115), suggesting that there is a direct effect between arginine intake and arginine oxidation. Arginine intake does not affect arginine synthesis in adult males (7); therefore, it appears that arginine homeostasis is controlled through the regulation of arginine oxidative catabolism (114).

1.2.6 *First-pass intestinal and splanchnic extraction of dietary arginine*

In adults, the small intestine is a major site of arginine extraction and metabolism. 33% of luminally-administered arginine was metabolized within the rat small intestine (64), and the major metabolic products of this metabolism included ornithine, citrulline, urea, CO_2 and proline (64). The ratio of plasma arginine fluxes when the arginine isotope was administered either intravenously or orally revealed that the splanchnic region of adult males extracted ~38% of dietary arginine (116). Assuming adult humans and rats have similar rates of arginine uptake by the splanchnic organs, combining the data from the two studies (64,116) shows that the intestine extracts more dietary arginine than the liver, which corresponds to the fact that the liver lacks the high affinity CAT-1, y^+ transporter (48).

In week-old enterally-fed piglets receiving an adequate-arginine diet, 40% of dietary arginine was extracted during first-pass intestinal metabolism (32). However, because the neonatal piglet intestine does not have high arginase expression (77,81), the metabolic fates of this absorbed arginine are unknown. It is unknown how dietary arginine intake affects splanchnic arginine extraction in either adult or newborn mammals.

1.2.7 *Arginine use in metabolic pathways*

1.2.7.1 Support of urea cycle function

The hepatic urea cycle is not considered an arginine catabolic pathway because for every mol of arginine that is converted to urea, one mol of ornithine is formed that can subsequently be used to reform arginine and perpetuate the cycle. If the urea cycle was completely efficient, it would not require an exogenous source of arginine because the cycle intermediates would be continuously regenerated. However, in species where arginine is an indispensable amino acid, arginine-free diets resulted in severe, life threatening hyperammonemia, which suggests that dietary arginine is needed to replenish urea cycle intermediates (34,117,118). Providing dietary arginine or ornithine restores urea cycle function in rats (94), dogs (93) and cats (92), further confirming that the cycle requires the replenishment of intermediates from exogenous sources. In the liver, arginine entering via the portal vein is used to support ureagenesis by acting as a precursor for ornithine, NAG, and citrulline, presumably largely via the action of arginase II in the mitochondrial matrix (112). The urea cycle has been extensively reviewed elsewhere (44,45,119,120), therefore only the key features will be briefly summarized.

The localization of the urea cycle to the periportal region of the liver is known as hepatic zonation [for extensive review, see (121)]. The enzymes CPS I, OTC, ASS and ASL are present in the periportal hepatocytes (121) and not in the perivenous cell types that have glutamine synthetase (121) and OAT activity (111). Therefore, the low affinity, high capacity urea cycle is located in a distinct area of the liver from the low capacity, high affinity glutamine synthetase, allowing for maximal ammonia detoxification within the liver (121). The other implication of this zonation is that ornithine formed via the urea cycle cannot be extensively catabolized because the OAT enzyme is not present, and therefore can be used to perpetuate the cycle (97,111). In piglets, the perivenous localization of glutamine synthetase was established by 1 week of age (122). Although there was CPS I expression throughout the liver in 1-wk old piglets, by 1 month of age it was localized to the periportal region, which is similar to adult pigs (122,123) and other mammals (123).

In a study of human livers obtained during autopsy, there was detectable activity of all urea cycle enzymes in all ages of livers studied (range of age from 12 wk gestational age to adult) (124). Therefore, fetuses and infants have the enzymatic capacity for urea formation. Prior to birth, the hepatic activity of OTC and arginase increased with increasing gestational age, and there was no effect of gestational age on any of the other enzymes studied (124). Fetal CPS, OTC, ASS and ASL activities were lower than postnatal values (124). Arginase activity was higher in fetal and premature infant livers than in the livers of newborns and infants in the first year of life; however, arginase activity in children and adults approached the pre-birth levels (124). Of all the enzymes, ASS activity was the lowest in the livers of humans of all ages, making the step catalyzed by ASS the rate limiting step for urea formation in humans (124), as had previously been described in rats (125).

The abundance of ORNT1 mRNA was measured in the livers of fetal mice (at 5, 3 and 1 days prior to the expected birth date) and in newborn mice up to 56 days of age (75). Before birth, there was a very low hepatic abundance of ORNT1 mRNA and it increased rapidly following birth until approximately 14 d of age (75). Combining this data with the human liver data, all of the elements of the hepatic urea cycle are present at the time of, or very shortly after birth.

In general, conditions that increase the amount of amino acids entering the liver also increase the activities of the hepatic urea cycle enzymes, in order to deal with the increased nitrogen load (reviewed in 119,120). Briefly, the hepatic activities of urea cycle enzymes increase with dietary protein intake (125,126) in adult mammals, with the notable exception of carnivores where there was a constitutively high expression of catabolic enzymes, regardless of protein intake (127). Starvation (125) and other catabolic conditions where muscle protein is broken down also increases hepatic urea cycle function (120). Urea cycle enzyme mRNA and protein expression are increased with glucagon, glucocorticoid and cAMP analog administration, with the exception of OTC which was only increased by glucagon infusion (reviewed in 120). Similar to the mRNA transcription of the urea cycle enzymes, the transcription of the ORNT1 mRNA was also induced by administration of glucocorticoids and cAMP (128).

Dietary arginine intake has an effect on plasma urea concentrations in neonatal piglets. During the short term (8 hour) administration of an arginine-free diet in neonatal piglets, the plasma urea concentrations declined, compared to baseline values in both parenterally and enterally-fed piglets, coinciding with increases in plasma ammonia values (34). However, after 5 days of the enteral administration of deficient or generous arginine diet, plasma urea concentrations were greater in piglets receiving the deficient arginine diet (33). An explanation for the discrepancy in these observations is that in the short term only urea cycle function was affected by the arginine-free diet, resulting in a decrease in plasma urea concentrations (34); however, after several days of inadequate dietary arginine intake, protein synthesis and breakdown may have been affected, although this was not directly measured, leading to an increase in amino acid catabolism and a greater need for urea synthesis.

Despite the presence of abundant expression of urea cycle enzymes in the liver, and the presence of OAT and proline oxidase (76) in the liver, there was virtually no hepatic release of arginine in adult rats (61,70,129) and humans (130), indicating that hepatic metabolism does not make a large contribution to whole-body arginine synthesis in adult mammals. One potential reason for the lack of contribution of the liver in whole-body arginine synthesis is the previously discussed hepatic zonation. In adult rats, the enzymes necessary for the formation of ornithine are present in the perivenous hepatocytes, while those necessary for the conversion of ornithine to arginine are in the periportal region (111,121). Therefore, during a single circulatory pass through the liver of adult mammals, arginine cannot be synthesized from precursor amino acids. Arginase activity is higher in the liver than any other tissue (131), which would also prevent the net synthesis of arginine by the liver. Finally, a study in permeabilized rat hepatocytes showed that there was a tight channelling of intermediates between the different cytosolic enzymes in the hepatic urea cycle (132). The channelling was particularly tight between ASL and arginase, and to a lesser extent between ASS and ASL (132). The implication of this channelling is that arginine produced by the liver is almost exclusively converted to urea and not released to the rest of the body (132).

1.2.7.2 Arginine as an allosteric activator of N-acetylglutamate

Arginine is an allosteric activator of NAGS, the enzyme responsible for the synthesis of N-acetylglutamate (NAG) from glutamate and acetyl-CoA (119). NAG is an essential co-factor for CPS I (133). In this sense, arginine is a regulator of its own synthesis and metabolism because it is needed for carbamoyl phosphate synthesis, which is required as an intermediate in both the urea cycle and in arginine synthesis. This metabolic function of arginine is another way that arginine can modulate ureagenesis: a certain level of circulating arginine may be necessary for optimal NAGS and subsequently CPS I activity. NAGS is a mitochondrial enzyme, and NAG concentrations were only detectable in the mammalian liver and the intestine (134), the two primary tissue sites of carbamoyl phosphate synthesis. Alternatively, it has also been shown using [^{15}N]-labeled arginine that arginine degraded to ornithine and subsequently converted to glutamate in the liver may be used as a precursor for the synthesis of NAG (112).

Studies conducted both *in vitro* and *in vivo* have produced inconclusive results with regards to the physiological significance of the role of arginine as an NAGS activator. *In vitro*, hepatic NAGS activity was stimulated by arginine addition to the incubation medium, as measured by mitochondrial NAG concentrations (135), NAG formation (136,137), citrulline formation (135) and the incorporation of [^{14}C]glutamate into NAG (136). Similar findings were also reported *in vivo*, with the intraperitoneal injection of 5 mmol/kg of arginine in rats resulting in transient increase (~50%) in hepatic NAG concentrations, with the magnitude and length of the stimulatory response being greater when glutamine was co-injected with the arginine (138). The incorporation of ^{14}C into hepatic NAG was much greater when arginine was injected simultaneously with the [^{14}C]glutamate (138), providing *in vivo* evidence that exogenous arginine administration can increase hepatic NAG synthesis.

The incubation of rat liver mitochondria with 0.1 mmol/L of arginine, a concentration more than twice the *in vivo* hepatic cytosolic concentration, only increased citrulline output by 11%, bringing into question the role of arginine in stimulating ureagenesis under physiological conditions (139). The reason suggested for the relatively low response in citrulline production to arginine addition was that mitochondrially-associated arginase may interfere with the transport of arginine into the mitochondria (139); however, further research demonstrated that arginine could be transported into rat liver mitochondria against a concentration gradient (140). A mitochondrial arginine concentration of 5-10 μmol/L is required for NAGS activation (138), and the mitochondrial arginine concentrations (140 – 340 μmol/L) exceed this by 20-40 times

(141); therefore, variations in arginine intake are not likely to be of physiological significance, although this has not been directly examined.

Thirty minutes after an NH_4Cl infusion in rats, the measured CPS I activity in the liver was no higher in rats that also received a simultaneous injection of 5 mmol/kg arginine than in those that received 5 mmol/L ornithine or no supplemental injection, suggesting that the endogenous levels of NAG were similar regardless of whether excess arginine was administered (142). However, CPS I activity was ~1-fold greater in rats that received an NH_4Cl versus a saline injection (142). In cats, a similar lack of response to arginine administration was observed, with cats receiving an arginine-free diet having similar rates of citrulline formation and liver mitochondrial NAG concentrations as cats receiving a complete diet (143). Increasing dietary protein resulted in an increase in hepatic citrulline formation in rats (135). The intraperitoneal injection of amino acids (0.5 – 3.0 g/kg) increased rat liver NAG content and CPS I activity; however, these increases were not affected by the omission of arginine from the injected amino acid solution (144). In pigs, the administration of somatotropin, a compound which enhances the efficiency of dietary protein use for protein accretion, decreased hepatic NAG and carbamoyl phosphate concentrations and decreased CPS I activity (145). Therefore, in a variety of mammalian species, hepatic nitrogen load appeared to have a much larger effect on CPS I activity than arginine availability. In general, the *in vivo* findings support the *in vitro* observations that arginine status may only have a very limited effect on NAGS and subsequently CPS I activity under physiological conditions.

However, a recent study in piglets showed that NAG concentrations had a profound effect on whole-body arginine status. In the enterocytes of growing piglets the synthesis of arginine from proline decreased dramatically from birth through 14 d of age (39,146), and this coincided with a drop in the jejunum mitochondrial NAG concentrations (146). It was proposed that NAG was limiting to *de novo* arginine synthesis in suckling piglets, and when N-carbamoyl glutamate (NCG), a stable analog of NAG was administered to piglets, the enterocyte rate of arginine synthesis increased several-fold, plasma arginine concentrations increased ~70% and piglet weight gain also increased, compared to saline administration (146). Therefore, although the physiologic importance of arginine for NAG synthesis is questionable, it is clear that NAG is critical for citrulline and subsequently arginine synthesis in piglets.

1.2.7.3 Nitric oxide synthesis

The discovery of arginine as the precursor of nitric oxide in mammalian cells is a fairly recent discovery (147). Arginine is converted to nitric oxide in most mammalian tissues via one of three NOS enzyme isoforms: eNOS (endothelial; type III NOS), iNOS (indicible; type II) and nNOS (neuronal; type I) (148). eNOS and nNOS tend to have relatively constant activities, both require Ca^{2+}/calmodulin as a co-factor and are collectively termed constitutive NOS (cNOS) (149). iNOS on the other hand, does not require Ca^{2+}, and is induced by certain cytokines and endotoxins (149). The kinetic features of the NOS enzymes have been recently reviewed (150) and will not be covered in this literature review. Some of the primary physiological roles of nitric oxide include: acting as a signalling molecule, the regulation of the vascular smooth muscle relaxation which leads to vasodilation, the proliferation of vascular smooth muscle cells, acting as a neurotransmitter, inhibiting the enzyme activity of enzymes with iron-sulfur centres (various mono-oxygenase and dioxygenase enzymes) , and mediating the immune response either directly through macrophage cytotoxicity or by promoting platelet aggregation, angiogenesis and thrombosis (reviewed in 41,45,148,149,151).

The activity of NOS *in vivo* may be affected by substrate availability (152) as has been shown in cultured macrophages where the inhibition of arginase resulted in an increase in arginine metabolism via iNOS (153). An increase in arginase activity may decrease the availability of arginine for nitric oxide synthesis; however, in mice where the circulating concentration of arginine was reduced using an infusion of arginase, there was no effect on nitric oxide synthesis from circulating arginine, which ranged from about 1.1 - 2.8 % of plasma arginine flux (154). More recently, the regulation of NOS activity by asymmetric dimethylarginine (ADMA), a metabolite generated during the turnover of methylated proteins (155,156), has been the topic of much research. ADMA is an endogenous inhibitor of NOS, and therefore elevated concentrations of this molecule are being touted as a risk factor for cardiovascular disease (reviewed in 151,156). ADMA may be degraded to dimethylamine and citrulline via the activity of dimethylarginine dimethylaminohydrolase (DDAH) (EC number 3.5.3.18) (156); however, this enzyme is inhibited by homocysteine (157), which is another risk factor for cardiovascular disease (158). Plasma homocysteine concentrations were increased in enterally-fed neonatal piglets due to either an increase in methionine intake or the absence of dietary cysteine (159); therefore, sulfur amino acid intake may be involved in the regulation of NOS activity, although this interaction has not been investigated in neonates.

In most nitric oxide producing cells, particularly those with iNOS activity, there is also ASS and ASL activity (44,160) that may be co-induced with iNOS activity both *in vivo* (161,162) and *in vitro* (163). Having ASS and ASL in NOS-expressing cells gives these cells the ability to regenerate the nitric oxide precursor, arginine, to sustain nitric oxide production (164). The citrulline-nitric oxide cycle has been recently reviewed in depth elsewhere (44,160,165).

In healthy adult males receiving a complete amino acid diet, nitric oxide synthesis represented 1.2% of whole-body arginine flux (166). In newborn infants with PPHN, nitric oxide synthesis was 0.15% and 0.47% of arginine flux for the infants during and following the acute pulmonary vasoconstriction of PPHN, respectively (15). In both of these studies (15,166), however, the arginine isotope was infused intravenously; therefore these data are a reflection of the peripheral use of circulating arginine for nitric oxide synthesis and the calculated use of arginine for nitric oxide synthesis may be different if the isotope is given enterally due to the effects of first-pass gut metabolism. The small intestine of young piglets has NOS activity (78) and based on arterio-venous difference studies in pigs (167) and mice (168), the portal drained viscera is a site of net nitric oxide production; therefore, if the arginine isotope is not exposed to first-pass intestinal metabolism, the whole-body conversion of arginine to nitric oxide may be underestimated.

There has been some research examining the effect of arginine intake on nitric oxide synthesis in mammals. In young rats receiving a diet that contained 0, 0.3 or 1% arginine, there was a dose-dependent effect of arginine intake on nitric oxide synthesis, as measured by nitrate excretion, both before (measure of constitutive nitric oxide synthesis) and following (measure of inducible nitric oxide synthesis) the injection of an endotoxin (169). However, neither cNOS nor iNOS activity in any of the tissues studied were affected by arginine intake (169). A study in enterally-fed neonatal piglets found that there was a tendency for arginine intake to affect plasma nitric oxide concentrations, and piglets receiving an arginine-deficient diet had a numerically lower plasma nitric oxide concentration than those receiving a generous intake of arginine (33). However, in the study in neonatal piglets (33), the actual rate of nitric oxide synthesis from arginine was not measured, and this may have been affected by arginine intake in a way that was not reflected in the plasma concentrations.

Nitric oxide and the effect of dietary arginine intake on its synthesis are of interest in neonates, particularly premature neonates, due to the suspected role of nitric oxide in the development of NEC and PPHN, two neonatal diseases that are seen primarily in premature infants. In a piglet model where NEC was experimentally induced in gut loops by the infusion of acidified casein, L-arginine administration was shown to have a protective effect, while N-omega-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, infusion did not prevent intestinal injury (19,170). These authors later demonstrated that the cNOS production of nitric oxide was protective against NEC (171), which is one possible mechanism whereby L-arginine administration reduced the incidence of NEC (13). Abnormal nitric oxide synthesis has also been implicated in infants with PPHN. There was a significant decrease in the conversion of arginine to urinary nitrate, which was used as an indicator of nitric oxide synthesis, in infants during the acute vasoconstriction of PPHN than when they were recovering (15), and the authors suggested that arginine availability may be a critical factor for nitric oxide synthesis in neonates. In cultured umbilical endothelial cells from infants, many infants (4 of the 6 infants studied) that were later afflicted with PPHN did not have the eNOS mRNA that could be amplified using polymerase chain reaction techniques, while all infants that did not develop PPHN had eNOS mRNA (172). Because of the role of arginine as a nitric oxide precursor, arginine infusion in infants with PPHN was investigated and was shown to improve infant oxygenation, based on an increase in arterial O_2 pressure (17). These results show that arginine, via its role as a nitric oxide precursor, is important in the prevention of the neonatal pathologies NEC and PPHN.

1.2.7.4 Creatine synthesis

Creatine is an important component for cellular energy metabolism and approximately 90% of the body's creatine is found in the skeletal muscle, with two thirds of this creatine being as phosphocreatine (173). Phosphocreatine acts as phosphate donor to ADP to generate ATP, although phophocreatine stores are depleted after a few seconds of intense exercise (173). The metabolic roles of creatine and the potential applications of creatine supplementation for exercise performance have been recently reviewed (173,174).

Arginine as an *in vivo* precursor for creatine has been known for some time, with the mechanism for creatine synthesis being proposed in the early 1940s (175). The synthesis of creatine involves the reaction of arginine with glycine to form guanidoacetate via the action of arginine:glycine aminotransferase (AGAT), and then the methylation of the guanidoacetate to form creatine, which is catalyzed by N-methyltransferase (45) (**Figure 1.1**, **Table 1.2**). AGAT protein has been identified in the proximal tubules of the kidney, liver hepatocytes and pancreatic acinar cells using immunochemistry techniques (176,177). The major organ site of guanidoacetate production was believed to be the kidney; however, a pancreatic perfusion study found that the pancreas could also produce physiologically significant quantities of guanidoacetate (177). The role of the liver in guanidoacetate synthesis cannot be ruled out because the livers of both humans and pigs have high AGAT activity, although the livers of laboratory rodents do not (174). Guanidoacetate synthesis by tissues other than the kidney is of physiological importance, because the renal release of guanidoacetate only accounted for 15-20% of creatinine excretion, therefore in order to keep tissues creatine levels constant, the remaining 80-85% of creatine must come from either dietary intake or endogenous synthesis by other tissues (178). Brosnan and Brosnan (178) recently proposed the 'arginine bicycle' in the liver which results in both creatine and urea synthesis, with ornithine as the common intermediate in both cycles. N-methyltransferase is expressed primarily in the liver, but other tissues such as the testes, pancreas and skeletal muscle also have detectable activity (174). The methylation portion of creatine synthesis is not believed to be limiting, under normal circumstances, for whole-body creatine synthesis (174).

In rats, supplemental creatine and guanidoacetate intake resulted in a downregulation of renal AGAT activity (179). Ornithine may also be a potential inhibitor of AGAT activity, as demonstrated by individuals with a genetic defect in ornithine metabolism having extremely high plasma ornithine concentrations and low tissue creatine concentrations (98,174). There has been little research concerning the effects of dietary arginine on creatine synthesis. However, early research in rat kidney slices found that under experimental conditions that increased arginine synthesis, such as the addition of citrulline, aspartate and glutamate to the incubation medium, there was also an increase in guanidoacetate formation (68), suggesting that renal arginine content does affect creatine formation to some extent.

1.2.7.5 Polyamine synthesis

The polyamines, putrescine, spermidine and spermine are small bioactive molecules that are essential for cell differentiation and proliferation (reviewed in 180,181). The immediate precursor for the polyamines is ornithine, and the rate limiting step in their formation is the step catalyzed by ODC (181) (**Figure 1.1**, **Table 1.2**). ODC activity is present in many tissues including the intestine, liver, kidney, thymus, brain and lungs, but the concentrations were greatest in rapidly proliferating tissues such as the intestine and thymus (182). The specific mechanisms whereby polyamines are involved with cell proliferation and differentiation are described in detail elsewhere (181).

In the neonatal enterocyte, ODC activity was high at birth, dropped sharply by 2 d of age and then gradually increased throughout the suckling period (183) (**Table 1.4**). Following weaning, the intestinal activity was ~65% higher than birth levels (78). ODC activity was induced by the post-weaning cortisol surge (184,185). The increased enterocyte polyamine concentrations were also associated with an increase in villus height, demonstrating the importance of intestinal polyamine synthesis in mediating intestinal adaptation that must occur following weaning (185). However, the supplementation of a piglet early weaning diet with a polyamine mixture (1 : 1.6 : 2.3 ratio of putrescine : spermidine : spermine) was detrimental to gut parameters including mucosal mass (186); therefore the ratio between the different tissue polyamines may be critical to their role in cell proliferation.

In order for arginine to act as a polyamine precursor, it must be hydrolyzed to ornithine via the action of arginase. Arginase I has been suggested to be the isoform required for polyamine synthesis, because similar to ODC it is also a cytosolic enzyme (187). In a study that used bovine epithelial cells transfected with either rat arginase I cDNA or mouse arginase II cDNA, it was found that basal arginase activity limited polyamine synthesis; however, there were no significant differences in putrescine and spermidine concentrations in cells transfected with arginase I versus arginase II (187). Therefore, mitochondrially-produced ornithine can be a substrate for cytosolic polyamine synthesis.

The effect of dietary arginine intake on the polyamine content in various murine tissues was recently investigated (182). There were no consistent changes in tissue polyamine content due to arginine supplementation, and the changes in concentrations that were observed, were relatively small. For putrescine, in the tissues where a response to diet arginine content was observed (ie. intestine), putrescine content decreased with increasing arginine intake, and these changes were apparent after 15 day of diet consumption (182). After 30 days of feeding the test diets, intestinal, kidney, muscle and thymus spermidine content increased and liver spermidine content decreased with increasing dietary arginine intake (182). Tissue spermine concentrations were largely unaffected by arginine intake (182). The physiological significance of these differences (182) in tissue polyamine content due to arginine intake, with respect to cell proliferation and differentiation, requires further investigation.

1.2.7.6 Agmatine synthesis

Agmatine was first identified in the bovine brain (188), and subsequent research identified that it was also present in many other mammalian tissues including the stomach, intestine, spleen and liver (189) and in the plasma of humans (190). Agmatine is synthesized from arginine via the action of arginine decarboxylase (ADC) (**Figure 1.1**, **Table 1.2**), a mitochondrial enzyme that has been identified in mammalian tissues such as the brain, kidney, liver, macrophages, macrophages and small intestine (191,192). However, research in piglet enterocytes from 0-58 d of age, did not detect any ADC activity (78), and it is unknown whether other neonatal tissues express ADC. Very little is known about the physiological roles and importance of agmatine in mammalian tissues, the importance of endogenous agmatine synthesis, or the effects of arginine intake on agmatine synthesis. Some of the suggested physiological roles of agmatine include: as a signalling molecule and a neurotransmitter, because it can bind α_2-adrenergic and imidazole receptors (188), as a putrescine precursor and a regulator of polyamine metabolism, and as a competitive inhibitor of NOS (reviewed in 193). Agmatine can be degraded by the enzyme agmatinase (EC number 3.5.3.11), and the resulting products are putrescine and urea; this could represent another metabolic pathway for polyamine synthesis. Agmatinase mRNA has been cloned from human tissues including the kidney, liver and skeletal muscle (194,195). However, despite the fact that some studies have detected ADC activity and agmatinase mRNA in human and rodent tissues, another study in rats, which used not only $^{14}CO_2$ production from [U-^{14}C]arginine, but also the production of [U-^{14}C]agmatine, did not detect any ADC activity or any conversion of [U-^{14}C]agmatine to putrescine (196). These authors (196) suggested that the ADC activity detected in other studies (191) was actually a result of the oxidative catabolism of arginine through ornithine, that the agmatine present in mammalian tissues was of dietary origin, and that the agmatinase that has been identified in human tissues (194,195) serves the physiological purpose of degrading dietary agmatine. A more recent study using [guanido-$^{15}N_2$]arginine and gas chromatography- mass spectrometry analysis to measure [guanido-$^{15}N_2$]agmatine appearance, concluded that in the rat liver there was agmatine synthesis from arginine (197). Therefore, the physiological significance and even the presence of mammalian ADC and agmatinase enzymes is a current topic of research controversy and further research is certainly required.

1.2.8 *Arginine requirements and intake*

1.2.8.1 Estimated metabolic arginine requirements in piglets and neonates

The current dietary arginine recommendations for arginine for a variety of species, established by the National Research Council and, in the case of humans, the Institute of Medicine for the National Academies, are provided in **Table 1.5**. There are presently no dietary requirements for arginine listed for humans of any age (198), based on the fact that healthy adults (5), children (4) and neonates (6) can maintain nitrogen balance without an exogenous source of arginine. Regardless of the dietary arginine requirement, all species have a metabolic requirement for arginine, for the metabolic functions discussed in section 1.2.7, and it is this requirement that must be met through

Table 1.5 Requirements for dietary arginine in different species during growth

Species	Size/age	Recommended dietary protein	Arginine requirement (% of diet)	Arginine requirement [g/(kg BW·d)]	Arginine requirement as a % of protein recommendation
Cat[1]	0.800 kg	22.5%	0.96	0.54	4.3
Chicken-broiler[2]	0-3 wk	23%	1.25	Not calculated	5.4
	3-6 wk	20%	1.10	Not calculated	5.5
Dog[3]	4-14 wk	22.5%	0.79	0.36	3.5
	> 14 wk, 5.5 kg	17.5%	0.66	0.30	3.8
Fish- channel catfish[4]	Not given	32%	1.20	Not calculated	3.8
Fish- rainbow trout[4]	Not given	38%	1.50	Not calculated	3.9
Fish- pacific salmon[4]	Not given	38%	2.04	Not calculated	5.4
Fish- common carp[4]	Not given	35%	1.31	Not calculated	3.7
Human[5]	0 – 6 months	1.52 g/(kg·d)	Not given	Not given	N/A
	7 – 12 months	1.2 g/(kg·d)	Not given	Not given	N/A
	1-3 years	1.05 g/(kg·d)	Not given	Not given	N/A
	4-8 years	0.95 g/(kg·d)	Not given	Not given	N/A
	9-13 years	0.95 g/(kg·d)	Not given	Not given	N/A
Rabbit[6]	Not given	16%	0.60	Not calculated	3.8
Rat[7]	Not given	15%	0.43	Not calculated	2.9
Swine[8]	4 kg	26%	0.59	0.37	2.3
	7.5 kg	23.7%	0.54	0.36	2.3
	15 kg	20.9%	0.46	0.31	2.2
	35 kg	18.0%	0.37	0.20	2.1
	65 kg	15.5%	0.27	0.11	1.7
	100 kg	13.2%	0.19	0.06	1.4

[1] Data are from (199) and represent the recommended arginine allowance using high quality feed ingredients. [2] Data are from (200) and represent the minimum arginine requirement. [3] Data are from (199) and represent the recommended arginine allowance using high quality feed ingredients. [4] Data are from (201) and represent the minimum arginine requirement for maximum performance in experimental conditions. [5] Data are from (198); protein requirements are the recommended dietary allowances; there are presently no human requirements provided for arginine. [6] Data from (202) and represent the minimum arginine requirement. [7] Data from (203) and represent the minimum arginine requirement. [8] Data from (36) and represent the minimum total arginine requirement.

the combination of dietary intake and endogenous synthesis. Therefore, if endogenous synthesis is compromised as in the case of intestinal disease, then the amount of arginine provided in diet must be increased in order to meet the metabolic demand.

Wu et al. (41) developed a factorial estimate of the daily metabolic arginine requirement in week-old piglets (2.5 kg). From their calculations, if piglets were gaining 200 g a day, this was equivalent to 27.2 g of protein and would require 1.88 g of arginine (204). Daily arginine use for creatine synthesis was estimated based on creatinine excretion [0.38 mmol/kg of creatinine excretion (205) = 0.95 mmol/d of arginine required for creatine synthesis] at 0.17 g, and arginine use for urea and nitric oxide synthesis was estimated based on the conversion of radio-labelled arginine (206) at 0.65 g/d. Therefore, the metabolic arginine requirement for a 2.5 kg piglet was estimated at 2.7 g/d or 1.08 g/(kg·d). This estimate does not include the amount of arginine required to replace arginine loss due to obligatory arginine oxidation, most likely because there were no available estimates for the quantitative importance of arginine oxidation to metabolic arginine requirements. However, this requirement is very similar to an the arginine requirement that was determined for parenterally-fed piglets, where endogenous arginine synthesis was assumed to be negligible, of 1.1 to 1.2 g/(kg·d), which was based on plasma ammonia and arginine concentrations and the rate of muscle protein synthesis (207). The metabolic arginine requirement is approximately 3 times the estimated dietary arginine requirement of growing piglets (**Table 1.5**) (36), showing that endogenous arginine synthesis generally meets a substantial portion of the metabolic arginine requirement.

There has been no formally established metabolic arginine requirement in neonates; however, there are a couple of methods that may be used to estimate this value. First, the same factorial method of Wu et al. (41) may be used to estimate human neonatal arginine requirements. Based on nitrogen balance data, neonates gain approximately 1.7 g/(kg·d) in protein (208) which, if arginine is assumed to be 7.7% of protein (**Table 1.1**). (25,26), would require 131 mg/(kg·d) of arginine. Based on creatinine excretion of 104 μmol/(kg·d) in week-old infants, and 95 μmol/(kg·d) in infants from 2-4 weeks of age (209), 16-18 mg of arginine is required daily for creatine formation. If it is assumed that ratio of arginine use for protein synthesis to the arginine use for urea cycle function and nitric oxide synthesis is similar in piglets and human neonates, then human neonates would require 45 mg/(kg·d) of arginine per day for urea cycle function and nitric oxide synthesis. Based on these values, the factorial estimate of the metabolic arginine requirement in human neonates is 193 mg/(kg·d). An alternate method of estimating arginine use in humans would be using the ratio of lysine to arginine requirement in piglets, and based on the lysine requirement in human neonates, calculating arginine requirement. In 3-5 kg piglets, the dietary lysine requirement is 0.94 (36), giving a lysine to arginine ratio of 0.81, assuming a metabolic arginine requirement of 1.15 g/(kg·d). The recommended adequate lysine intake in human neonates in 107 mg/(kg·d) (198), which would give an estimated arginine requirement of 132 mg/(kg·d). There is a very large discrepancy between these two estimated arginine requirements for human neonates, possibly because human neonates grow much more slowly than neonatal piglets and therefore the ratio of lysine to arginine required may not be the same in neonatal humans and piglets. It is clear that research focused specifically on the arginine requirement of neonates is required.

1.2.8.2 Estimated arginine intake and endogenous synthesis in piglets and neonates

The estimated intake of arginine from sow's milk in week-old piglets is 1.06 g/d, or 0.42 g/(kg·d), based on 0.78 L of milk consumed per piglet, an arginine content of 1.43 g/L (90), and a digestibility of 95% for the milk proteins (41). From the estimation of daily arginine use by piglets in section 1.2.8.1, dietary intake from milk only accounts for approximately 35-39% of the metabolic arginine requirement, illustrating the importance of endogenous arginine synthesis in maintaining arginine balance. Using week-old enterally-fed piglets receiving the lowest amount of arginine that could be fed without causing hyperammonemia, the maximum rate of endogenous arginine synthesis was calculated at 0.68 g/(kg·d) (33). Therefore, if suckling piglets are endogenously synthesizing arginine at the maximum rate, the metabolic arginine requirements can be met. However, 7-21 d old piglets receiving a milk-replacer formula providing 0.51 g/(kg·d) of arginine, a value slightly higher than the estimated intake from sow's milk (41), did not grow as rapidly as piglets receiving either 0.69 or 0.82 g/(kg·d) arginine, showing that in suckling piglets the combination of dietary arginine intake and endogenous synthesis was not enough to sustain maximal piglet growth (14). Therefore, assuming the metabolic requirement for arginine is 1.08 – 1.20 g/(kg·d), then it does not appear that suckling piglets are able to synthesize arginine at the maximum rate, due to some limitation in the metabolic pathway that requires further investigation.

In the parenterally-fed piglet model, where endogenous arginine synthesis is assumed negligible (34), the targeted intake of amino acids is 15 mg/(kg·d) (30), which would result in a range of arginine administration from 0.71 g/(kg·d) (Vamin) to 1.83 g/(kg·d) (Trophamine) (21), depending on the parenteral solution used (**Table 1.1**). Therefore, not all parenteral solutions would provide enough arginine to meet the daily arginine use of growing piglets (41).

Breast-fed Swedish infants, 2-5 months of age, consume 0.84 L of breast milk each day, with a total amino acid composition of 8.7 g/L (210). With a milk arginine content of 36 mg arginine/ g total amino acids (24) (**Table 1.1**), and assuming an average infant weight of 6 kg, for this age range, this represents an arginine intake of 44 mg/(kg·d). Therefore, in human breast-fed infants, endogenous arginine synthesis must be responsible for 67-84% of the daily arginine used to meet metabolic requirements (section 1.2.8.1).

The premature human neonate requires 2-3 g/(kg·d) of protein for maximum rate of growth (9,20); however, in low birth weight infants (< 1000 g), protein may be provided at intake rates in excess of 5 g/(kg·d). Therefore, from **Table 1.1**, the parenteral infusion of the various amino acid solutions at this targeted rate of intake will provide anywhere from 94 – 336 mg/(kg·d) arginine, which is a huge range of possible intakes. The lower intakes only provide ~50% of the estimated metabolic requirement (section 1.2.8.1). The pediatric infant formula, Similac Special Care [63 mg arginine/100 mL formula (13); 1.66 g protein/100 mL formula (20)], at 2-3 g/(kg·d) protein intake will provide 75 – 114 mg/(kg·d), which although greater than the arginine intake for breast milk, is still below the metabolic arginine requirement. Therefore, in order to meet estimated requirements, premature and breast-fed infants are very dependent on endogenous arginine synthesis; however, there has not been any research in infants, either healthy or ill, to determine whether metabolic requirements for arginine can be met by endogenous synthesis and what the effects of neonatal illness are on endogenous arginine synthesis.

1.2.9 *Possible implications of excess arginine intake*

Although for the previously discussed reasons it is critical to provide adequate dietary arginine in neonates, due to the role of arginine as a precursor for a number of bioactive compounds, another very important consideration may be the maximum recommended level of dietary arginine intake. A maximum recommended dietary arginine intake has not been demonstrated for either humans or pigs at this time; however, there is some evidence that too much dietary arginine may have adverse consequences.

In neonatal piglets, the twice daily oral bolus administration of arginine (0.12 g/kg) resulted in a decrease in plasma lysine and histidine concentrations, relative to piglets receiving either no supplement, water or alanine administration (146), which could have the consequence of decreasing rates of gains due to a limitation, in particular, in lysine, due to lysine-arginine antagonism. However, when the arginine was added to the milk-replacer, and not given as a twice-daily bolus dose, there was no effect of arginine intake on plasma concentrations of either histidine or lysine (42). Therefore, it is unclear whether the well-described antagonism between arginine and lysine (reviewed in 211) is an important consideration in diet formulations for neonates. In older pigs, implications of the antagonistic relationship between lysine and arginine has been well studied because lysine is typically the first limiting amino acid in commercial diets, and arginine content of the diet is well in excess of the NRC requirements (36,212); therefore, studies also examined whether the high arginine intake had detrimental effects on pig growth (213-215). For pigs, there was no apparent effect of high lysine on arginine metabolism (216), or high arginine intake on lysine metabolism (213-215); therefore, there is no apparent lysine-arginine antagonism to consider when formulating growing-finishing swine diets.

In healthy infants and children that were given 0.5 g/kg of arginine-HCl over 30 minutes, plasma arginine concentrations increased dramatically as did urea excretion, but no other adverse consequences were observed, as this arginine was cleared from the blood over several hours (217). In infants with an inborn error affecting the arginase gene, the consequences associated with the inability to degrade arginine are devastating and include spastic tetraplegia, seizures and psychomotor retardation, and these conditions become progressively worse over time, although the mechanisms responsible for these symptoms have not been elucidated (218). Therefore, there are potential adverse consequences to extremely elevated plasma arginine concentrations in neonates.

In adult patients, the beneficial effects of supplemental arginine administration have not been conclusively shown (reviewed in 219), and in certain inflammatory conditions supplemental arginine may even be harmful (220). In children with sepsis, arginine synthesis was not high enough to replace the amount of arginine converted to urea and nitric oxide, and arginine became an indispensable amino acid (221). However, sepsis is characterized by the induction of iNOS activity and during sepsis nitric oxide synthesis increased (221). Excess nitric oxide production is associated with many of the clinical manifestations of sepsis including hypotension, excessive inflammation, liver damage and vascular permeability (220); therefore, in sepsis there is the dilemma of whether or not to supplement arginine. Further research is required to determine the optimal range of arginine intake, under a variety of physiological and pathological conditions in humans of all ages. A complete knowledge of arginine intake, requirements and metabolism in neonates is required to ensure that adequate arginine is provided to ensure optimal growth and prevent neonatal pathologies while minimizing the risk of the consequences associated with excessive arginine intake.

1.2.10 *Arginine metabolism in individuals and species with unique aspects of arginine metabolism*

1.2.10.1 Arginine metabolism in humans with genetic disorders affecting the arginine biosynthetic and metabolic pathways

Genetic defects, resulting in errors in metabolism, have been described in humans for virtually every enzyme involved in arginine synthesis and metabolism, as shown in **Table 1.6**. For the scope of this discussion, only the urea cycle disorders of the CPS I, OTC, ASS, ASL, NAGS and arginase enzymes will be discussed as these have been shown to have specific effects on circulating arginine levels (218,222,223). The presenting symptom for all of these conditions, with the exception of the arginase deficiency, is hyperammonemia with visible symptoms including lethargy, vomiting and requiring stimulation for feeding; however, there are many other biochemical markers in both the plasma and the urine that can be used to differentiate between the different

Table 1.6 Summary of the genetic disorders affecting arginine metabolism[1]

Enzyme/transport protein involved	Biochemical indicators used to diagnose the disorder and other symptoms
Arginase	- Abnormally high: plasma arginine and ammonia concentrations, urinary orotate excretion - Abnormally low: serum urea concentrations - Other symptoms include spastic tetraplegia, seizures, psychomotor retardation, hyperactivity, growth failure
Argininosuccinate lyase	- Hyperammonemia (usually mild) - Abnormally high: plasma citrulline and argininosuccinate concentrations - Abnormally low: plasma arginine and serum urea concentration
Argininosuccinate synthetase	- Hyperammonemia - Abnormally high: plasma citrulline concentrations - Abnormally low: plasma argininosuccinate and arginine concentrations, low serum urea concentrations
Carbamoyl-phosphate synthetase	- Hyperammonemia (severe, often lethal) - Abnormally low: plasma citrulline and arginine concentrations, serum urea concentrations - Normal: urinary orotate excretion
Guanidinoacetate N-methyltransferase	- Abnormally high: plasma and cerebrospinal fluid guanidoacetate concentrations, urinary guanidoacetate excretion - Abnormally low: plasma and cerebrospinal fluid creatine concentrations, urinary creatinine excretion - Other symptoms: muscular weakness, neurological symptoms, poor head control, austic-like behavior
N-acetylglutamate synthase	- Hyperammonemia - Plasma ammonia concentrations reduced by the administration of an NAG analog, N-carbamoylglutmate - Abnormally high: plasma glutamine concentrations - Abnormally low: plasma citrulline concentrations - Normal: orotate excretion, CPS I activity
Ornithine aminotransferase (Gyrate atrophy of the choroids and retina)	- Abnormally high: plasma, urine, cerebrospinal fluid and aqueous humor ornithine concentrations - Abnormally low: plasma creatine concentrations, muscle phophocreatine content - Normal: plasma ammonia concentrations - Other symptoms include muscular and retinal abnormalities
Ornithine transcarbamoylase	- Hyperammonemia - X-linked disorder; most common disorder affecting arginine metabolism - Abnormally high: urinary orotate excretion - Abnormally low: plasma citrulline and arginine and

	serum urea concentrations
Ornithine transporter-1 (Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome)	- Hyperammonemia - Abnormally high: plasma ornithine concentrations, urinary homocitrulline and orotate excretion
P5C dehydrogenase (Type II hyperprolinemia)	- Abnormally high: plasma proline concentrations, urinary proline, P5C, hydroxyproline and glycine excretion - Does not appear to have any negative effects on health
Proline oxidase (Type I hyperprolinemia)	- Abnormally high: plasma proline concentrations, urinary proline, hydroxyproline and glycine excretion - Abnormally low: urinary P5C excretion - Does not appear to have any negative effects on health

[1] Data from this table adapted from the information provided in references (98,218,222-226)

metabolic disorders (**Table 1.6**). Although plasma ammonia concentrations may be elevated in individuals with an arginase deficiency, symptoms of hyperammonemia are not generally observed, although there are other neurological pathologies (**Table 1.6**) (218,222). For NAGS deficiency, the symptoms can be alleviated by the administration of a stable NAG analog called NCG, which is able to activate CPS I, and restore urea cycle and arginine synthetic function (223,227). In addition, NCG administration may be a useful diagnostic tool in distinguishing between CPS I and NAGS defects, because both defects have similar symptoms, but only NAGS errors can be corrected by NCG administration (227).

These disorders frequently present in the first few days of life (218) and ensuring the proper nutrition of these neonates is of critical importance, specifically because arginine must now be considered as an indispensable amino acid and because the intake of protein must be limited to prevent excess ammonia production from amino acid catabolism. Therefore, to ensure optimal nutrition of human infants with urea cycle disorders, it is critical that the metabolic requirement of arginine is known and that the dietary requirements of all indispensable amino acids are also known. With knowledge of the arginine and indispensable amino acid requirements, low protein diets, that still contain adequate amounts of non-protein energy, with an optimum balance and content of the indispensable amino acids, can be fed to these infants. The use of such diets will prevent excess ammonia production from amino acid catabolism and will promote net protein synthesis. In the case of an arginase deficiency, the ability for endogenous arginine synthesis remains intact and it may be beneficial to feed a low arginine diet;

however, experimental assessment of the benefits of low arginine diets have produced inconclusive results due to differing nitrogen intakes (218).

Individuals with these inborn errors in metabolism (**Table 1.6**) can also provide us with valuable information concerning different aspects of arginine metabolism. Individuals with errors in NAGS, CPS I, OTC, ASS and ASL have an absolute dietary requirement for arginine for protein, nitric oxide, polyamine and creatine synthesis, but not for urea synthesis (218). Therefore, if the metabolic requirement for arginine of normal, healthy individuals is compared to the metabolic requirement of individuals with these urea cycle disorders, the difference in these requirements would be equivalent to the metabolic requirement of arginine for urea cycle function. The urea excreted in the urine of affected individuals is due to the breakdown of dietary or circulating arginine, and not due to the conversion of ammonia to urea via the urea cycle, and therefore the rate of urea excretion in these individuals may be used as an indicator of whole-body arginine degradation.

In some cases, patients with urea-cycle related disorders receive liver transplants to prevent hyperammonemia and its related consequences, while enabling them to eat a higher protein diet (228). Although these patients now have a complete hepatic urea cycle, the affected enzymes are still missing from the intestine and kidney, and therefore these individuals continue to have a dietary requirement for arginine (222,228). In fact, individuals with CPS I and OTC defects who received liver transplants had lower plasma arginine concentrations after the transplant than before the transplant (222). This supports the intestinal-renal axis as being the major site of synthesis for circulating arginine, with hepatically-derived arginine being used within the liver to support urea cycle function.

1.2.10.2 Arginine metabolism in cats

Cats fed an arginine-free diet became rapidly hyperammonemic (117) demonstrating that arginine is an indispensable amino acid in cats. Although ornithine supplementation to an arginine-free diet restored urea cycle function in cats (117), as indicated by lower plasma ammonia concentrations, plasma arginine concentrations and growth remained lower (92,117) than in cats receiving an arginine-containing diet. Citrulline supplementation, however, was able to restore plasma arginine concentrations and growth rate (92). Therefore in these cats, there was an inability to synthesize sufficient ornithine to support urea cycle function, which then limited the formation of citrulline that could be used for arginine synthesis. On a per kg body weight basis, the small intestinal mucosa of cats only had 5% of the P5C synthase activity (229) of the rat intestinal mucosa. Furthermore, the activity of OAT per g mucosa in cats was only 23% of the activity in rats. The very low intestinal activities of these enzymes in cats (229) places a severe limitation on the *de novo* rate of synthesis of ornithine, and subsequently citrulline; this provides a metabolic explanation for why arginine is an indispensable amino acid in cats. The expression of proline oxidase (EC number 1.5.99.8) has not been investigated in the cat; however, even if there is measurable activity of this enzyme in feline tissues, it appears unlikely that proline makes a large contribution to whole-body arginine synthesis due to the low OAT activity.

Because intestinal citrulline synthesis is virtually absent, cats have much lower plasma citrulline concentrations (> 10 μmol/L) (230) than rats, rabbits or pigs (60-120 μmol/L) (95,230). These low plasma citrulline concentrations may be a reason for the limited amount of renal arginine synthesis from citrulline in cats (230). Although the cat kidney, in particular the proximal convoluted tubule, can convert radio-labelled citrulline to arginine (230), with the physiological citrulline concentrations, the daily estimated rate of this conversion was only approximately 86 μmol/day (0.015 g/d) in adult cats (230) versus over 350 μmol/day (0.06 g/d) in rats fed a standard diet (71). However, in kittens fed an arginine-free diet with supplemental citrulline, plasma citrulline concentrations were ~250 μmol/L and plasma arginine concentrations were not different from kittens receiving an arginine-sufficient diet (92). These observations suggested that there was an increase in renal arginine synthesis with increased plasma citrulline concentration (92), which is in agreement with rat data (71). Therefore in cats, renal arginine synthesis is low due to low circulating citrulline concentrations.

Because mature 2 - 2.5 kg cats only synthesized approximately 0.015 g [0.007 g/(kg·d)] of arginine each day (230), they relied on their diet provide at least 94% of the arginine requirement [0.12 g/(kg·d)] (199); therefore, the arginine requirement of the cat represents the amount of arginine used for protein synthesis, the support of urea cycle function and for the synthesis of other metabolites including creatine, polyamines and nitric oxide. In comparison to other, non-carnivorous, ureotelic species, cats have a very high arginine requirement, as a % of diet, this is reflective of the fact that endogenous synthesis is low. In addition, cats consume a very high protein diet (**Table 1.5**), which requires a large amount of arginine to support urea cycle function. The hepatic activities of many enzymes involved in amino acid degradation, remain high during low protein intake and starvation (127), meaning that amino acid degradation is constitutively high, compared to non-carnivores, and that a more active urea cycle is necessary to detoxify the resulting ammonia. The relatively unchanging activities of hepatic catabolic enzymes (127) may explain the high crude protein and arginine requirements of the cat. However, the arginine content of most meat ingredients ranges from ~2-5% (231); therefore, in cats receiving a typical carnivore diet arginine intake is usually adequate.

Although when cats are fed an arginine-free diet they display many of the same symptoms as humans with inborn errors in urea cycle metabolism, they are not an appropriate experimental model for these disorders (92,117,218,222). The main reason for their unsuitability is due to the fact that urea synthesis in cats can be restored, and plasma ammonia concentrations returned to normal, if ornithine, citrulline or arginine (92) is added to the diet; however, in humans, ammonia conversion to urea cannot be restored through dietary manipulation. This is because in cats, unlike in humans with urea cycle disorders, the limitation in urea synthesis does not involve the urea cycle enzymes, but the enzymes responsible for producing ornithine from glutamate (229) **(Figure 1.1**, **Table 1.2**).

1.2.10.3 Arginine metabolism in poultry

The dietary essentiality of arginine in the diet of chicks was first demonstrated in the 1930's by studies that showed that adding arginine to chick diets either low or devoid of arginine improved chick growth (232,233). Further investigation revealed that the reason that arginine was a dietary indispensable amino acid in chicks was the lack of detectable activity of the enzyme CPS I in all of the tissues that were investigated, including the liver, kidney, pancreas and spleen (234). In the chick kidney, but not liver, there was, however, detectable activity of the enzymes OTC, ASS and ASL, but in comparison to the activity found in the liver of rats (125), the renal activites of these enzymes in the chick were low. However, even the low renal ASS and ASL activities were of physiological significance, as citrulline addition to an arginine deficient diet was equally as effective at promoting chick growth as the addition of an equivalent amount of arginine (235). The ability of citrulline to spare the arginine requirement in chicks was further confirmed when the label from [ureido-^{14}C]citrulline was found in chick body protein as arginine (236). Neither ornithine (233,235,236) nor bicarbonate (236) were precursors for arginine in chicks, in support of the enzymatic data finding no detectable CPS I activity (234).

The nutritional significance of the enzymatic data in chicks is that there is a dietary requirement of arginine for chicks. Arginine in chicks is only used for protein synthesis and the synthesis of metabolically-important molecules such as creatine. Creatine concentrations are related to arginine intake and availability in chicks (237-239), and therefore although it is likely a small percent of dietary arginine that is used for creatine synthesis, creatine synthesis still accounts for a portion of the arginine requirement. Unlike mammals, the metabolic arginine requirement of chicks does not include the support of urea cycle function. The main nitrogenous waste product in birds is uric acid and its formation is not arginine-dependent; therefore, the urea cycle is not necessary for nitrogenous waste excretion and there is an almost complete lack of urea cycle (which are also arginine synthetic) enzymes in the chick liver (234).

Although chicks are incapable of arginine synthesis, there is the enzymatic capacity for arginine degradation via arginase activity. However, unlike mammals, the arginase activity of the kidney is ~30 times higher than in the liver (234). Although there is no functional urea cycle in chicks, chicks do excrete some urea and this is a quantitative measure of arginine degradation. Studies in laying hens showed that renal arginase activity and urea excretion were both increased by increasing arginine or protein intake (240). Furthermore, urea production was related to plasma arginine concentration (240). Therefore, similar to other animals, arginine concentrations are partially regulated by arginine degradation in chickens. However, although arginine administration increased urea excretion in hens, the same was not observed with ornithine infusion (240), which provides further support for ornithine being an ineffective arginine precursor in poultry. Studies in chicks showed that when renal arginase activity was altered by either changing dietary factors (increasing either lysine, arginine or tyrosine content of the diet) or by using strains of birds that were selected to have different levels of renal arginase expression, that as arginase activity increased, there was also an increase in the arginine requirement (241). Therefore, the dietary requirement for arginine in chicks (**Table 1.5**) represents the metabolic arginine requirement for protein and metabolite synthesis as well as to replace the arginine that is degraded by renal arginase.

1.2.10.4 Arginine metabolism in fish

Arginine is a dietary indispensable amino acid in fish. Similar to chicks, the limitation in arginine synthesis is likely the rate of synthesis of carbamoyl phosphate. Unlike in mammals, the enzyme that catalyzes the synthesis of carbamoyl phosphate for urea cycle function is carbamoyl phosphate synthetase III (CPS III; EC number 6.3.5.5), which similar to CPS I is mitochondrial and requires NAG as a co-factor, but instead of ammonia as its substrate, it requires glutamine (242). Studies in rainbow trout have shown that there is no hepatic activity of CPS III and very low hepatic OTC activity (243). The muscle of trout has the greatest activity of CPS III (243); however, in comparison to the activity of CPS I in rat liver (125), its activity is very low [300 µmol of product formed/(g liver·h) in rats vs. 0.0144 µmol of product formed/(g·muscle·h) in rainbow trout], and thus there is a very limited capacity for carbamoyl phosphate and subsequently *de novo* arginine synthesis in fish. There is detectable activity of all of the other enzymes involved in arginine synthesis in the tissues (specifically liver, kidney, muscle and intestine) of rainbow trout (243,244).

There is some isotopic evidence that trout are capable of some *de novo* arginine synthesis. Injection of [1-^{14}C]ornithine into fish resulted in approximately 10% of the label being recovered in arginine (244). In order for ornithine to be used for arginine synthesis, there must be carbamoyl phosphate formation; therefore, although the activity of CPS III may be low (243), it may still be of physiological relevance. However, *de novo* arginine synthesis was not sufficient to sustain optimal trout growth because the addition of an equimolar amount of either arginine or citrulline to a low-arginine basal diet resulted in greater rates of gain, better feed efficiency and higher plasma arginine concentrations than the addition of either glutamate or ornithine (244).

Despite the low capacity of the fish urea cycle, there is some urea production, but this is largely believed to be due to the degradation of dietary arginine (245,246). Arginase activity, primarily mitochondrial (243,247), has been detected in the liver, kidney, muscle and intestine of rainbow trout (243,244), with the kidney, followed by the liver, having the greatest activity of arginase per gram tissue (243,244). In rainbow trout, hepatic arginase activity was not affected by arginine intake (247); however, in turbot fish, there was a strong correlation between arginine intake and urea production, which the authors concluded was due to increasing hepatic arginase activity (245). Therefore, in fish, the relationship between arginine intake and whole-body arginine degradation via arginase activity, may be species-specific. The extra-hepatic contribution to arginine degradation requires further investigation.

1.3 Total parenteral nutrition in piglets

1.3.1 *Effects of parenteral nutrition on the piglet intestine*

A number of morphological and functional changes occur in the small intestine of piglets during parenteral feeding. In particular, parenteral nutrition resulted in a marked intestinal atrophy characterized by lower small and large intestinal weights compared to sow-fed, formula-fed and elemental diet-fed piglets (22,248). Compared to the enteral administration of an elemental diet, parenteral feeding of an identical diet resulted in the significant decrease in duodenal, jejunal, and ileal mucosal and total tissue weight (22). In addition, the villus height and crypt depth of the duodenum, jejunum and ileum were also reduced as a result of parenteral versus enteral feeding (22,249), and these differences were observed regardless of whether the enteral diet was sow's milk (249) or an elemental diet (22). These intestinal differences due to parenteral feeding resulted in a decrease in absorptive surface area in both the jejunum and the ileum (249). Intestinal atrophy in parenterally-fed piglets can be observed within 24 hours of the initiation of parenteral feeding (79). Differences in intestinal weight and histology were also observed in 4 wk old parenterally-fed piglets (250), and therefore appear to be independent of piglet age.

One reason for the changes in intestinal morphology during parenteral feeding is that intestinal protein turnover and cell proliferation rates are affected by route of feeding. Total mucosal protein synthesis was lower in parenterally than enterally-fed piglets (251). Parenteral feeding decreased the absolute rate of protein synthesis in the jejunum and proximal ileum, and these differences were more pronounced in the proximal regions of the small intestine than in the distal regions (252). The absolute rate of protein degradation was greater in enterally than parenterally-fed piglets in the jejunum and proximal ileum (252). However, in parenterally-fed piglets, but not enterally-fed piglets, the rate of protein degradation exceeded the rate of protein synthesis, resulting in a negative protein balance in the jejunum and proximal ileum of parenterally-fed piglets (252). An enteral intake of 40 - 60% of total nutrient intake was required to sustain the enteral rates of intestinal protein synthesis and cell proliferation, and prevent the parenteral-feeding induced intestinal atrophy (252,253). Rates of jejunal and ileal cell proliferation were also lower in parenterally-fed piglets (249); therefore, in neonatal piglets the intestinal atrophy is associated with both a reduction in intestinal cell numbers (hypoplasia) and a decrease in cell protein synthesis (hypotrophy).

Portal vein blood flow was similar in parenterally-fed piglets as in fasted piglets, and was ~40% lower than the portal vein blood flow in enterally-fed piglets (79). Superior mesenteric artery blood flow was ~80% of the fasting blood flow value in parenterally-fed piglets as compared to ~130% of the fasting value in enterally-fed piglets (79). When piglets were changed from enteral to parenteral feeding, blood flow dropped immediately with rates stabilizing 4-8 hours after the onset of parenteral feeding (79). A major implication of this decreased intestinal blood flow as a result of parenteral feeding, is that in addition to the absence of luminal nutrients, the intestine is also exposed to fewer arterial nutrients per unit time, for possible uptake and metabolism. Therefore, in the case of arginine, if the arterial metabolism of circulating proline does make an important contribution to whole-body arginine synthesis, then the reduction in blood flow due to parenteral feeding would decrease the amount of proline that circulates through the intestinal tissues each minute, which may reduce proline uptake and conversion to arginine.

Clearly parenteral feeding results in numerous morphological and functional changes that almost certainly affect intestinal and whole-body amino acid metabolism. Arginine metabolism, in particular, may be adversely affected by parenteral feeding, due to the importance of the small intestine in endogenous arginine synthesis (33,37). This was confirmed by the findings of Brunton et al (34) which showed that there was a negligible amount of endogenous arginine synthesis in parenterally-fed piglets.

1.3.2 *Glucagon-like peptide 2 administration in parenterally-fed neonatal piglets*

Glucagon-like peptide 2 (GLP-2), is a 33 amino acid proglucagon-derived peptide that has intestinotrophic properties (reviewed in 254). GLP-2 is synthesized and released by the L-cells of the distal ileum, and the proximal small intestine has the greatest receptor expression, although other tissues such as stomach, brain and lungs also have GLP-2 receptors (254). In the intestine of piglets, the GLP-2 receptor was found in both the myenteric neurons and the enteroendocrine cells of the villus epithelium, and was co-localized with eNOS, serotonin and vasoactive intestinal polypeptide (255). Plasma GLP-2 concentrations of parenterally-fed piglets (32 pmol/L) were ~40% of the concentrations observed in sow-fed piglets (75 pmol/L) (256). In human neonates following intestinal resection, postprandial plasma GLP-2 concentrations were closely related with the length of the small intestine ($r^2 = 0.75$), and were related with the clinical outcome: of the 4 infants with postprandial GLP-2 concentrations less than 14 pmol/L, only one infant survived (257).

The simultaneous infusion of GLP-2 [25 nmol/(kg·d)] with a parenteral diet reduced the degree of intestinal atrophy, compared to parenteral feeding alone (249). Parenterally-fed piglets receiving the GLP-2 had increased jejunal and ileal mucosal mass, increased villus height in the jejunum and crypt depth in both the jejunum and ileum, and increased small intestinal absorptive surface area, compared to parenterally-fed piglets (249). In most instances, the intestinal morphological parameters were not different in the GLP-2 treatment group than in piglets that received sow's milk (249). A recent study by Burrin and collegues (256) found that although there was a dose dependent response between the rate of GLP-2 infusion and plasma GLP-2 concentrations in parenterally-fed piglets, improvements in gut morphology did not occur at the lowest [2.5 nmol/(kg·d)] rate of GLP-2 infusion, despite the fact that plasma concentrations were well above the normal physiological range for enterally-fed piglets (~200 vs. 75 pmol/L). GLP-2 infusion, at a rate of 10 nmol/(kg·d), in parenterally-fed piglets resulted in increased intestinal cell proliferation and protein synthesis, compared to parenterally-fed control piglets and those piglets receiving lower rates of GLP-2 infusion, although cellular apoptosis was suppressed at all levels of GLP-2 infusion (256). In addition, the intestinal activities of enzymes associated with apoptosis, caspase-3 (EC number 3.4.22.B9) and caspase-6 (EC number 3.4.22.B11) were also dose-dependently suppressed by increasing rates of GLP-2 infusion (256). However, compared to enterally-fed piglets, parenterally-fed piglets receiving a GLP-2 infusion [25 nmol/(kg·d)] had lower rates of intestinal protein synthesis, proliferation and degradation, although rates of intestinal apoptosis were similar (249).

The fractional rate of intestinal protein synthesis increased with increasing plasma GLP-2 concentration in a non-linear manner, and piglets receiving the 10 nmol/(kg·d) had a significantly greater rate of intestinal protein synthesis than piglets not receiving GLP-2. GLP-2 infusion [10 nmol/(kg·d)] did not increase protein synthesis or total DNA content of other splanchnic organs, including the spleen, stomach and liver (256). This confirms that the small intestine is the primary site of GLP-2 action *in vivo*.

Acute GLP-2 infusion in ~3 wk old piglets, parenterally-fed for 7 days, resulted in a 25% increase in portal blood flow within 10 minutes of the initiation of GLP-2 infusion (258). This increase in blood flow was abolished by the co-infusion of L-NAME with GLP-2, indicating that the effects of GLP-2 on blood flow are nitric oxide dependent (258). In addition, acute GLP-2 infusion also increased intestinal eNOS protein expression (256,258) relative to the saline infusion, and levels were still higher after a week of continuous GLP-2 infusion at a supraphysiological [10 nmol/(kg·d)] rate (256). The proximal regions of the small intestine had a greater increase in blood flow than the distal small intestine and colon (259), and was shown to be due primarily to increases in superior mesenteric artery blood flow (259). In addition, the serosal blood flow (+~100%) increased to a greater extent than mucosal blood flow (+~50%) following GLP-2 administration (259), which may be related to the fact that myenteric neurons express the GLP-2 receptor (255).

GLP-2 infusion in parenterally-fed increased the portal-drained viscera extraction of all of the indispensable amino acids and glutamine from arterial circulation (258). Arginine was not extracted by the portal drained viscera, regardless of GLP-2 administration, although there was a numeric increase in arginine output by the portal-drained viscera in piglets receiving the GLP-2 infusion (258). The increased portal-drained viscera uptake of indispensable amino acids may have implications for their requirements. Parenterally-fed piglets have lower requirements for threonine (260), methionine (261) and the branched chain amino acids (262) than enterally-fed piglets, although the effects of GLP-2 administration on amino acid requirements have not been investigated. Therefore, by increasing intestinal blood flow and improving intestinal morphology, GLP-2 administration may alter whole-body amino acid requirements and metabolism, compared to parenteral-feeding alone. GLP-2 infusion in parenterally-fed piglets also has the potential to be a useful experimental tool to study the effects of healthy gut morphology on amino acid metabolism using a parenterally-fed piglet model.

1.4 The use of a neonatal piglet model for the human neonate

Appropriate nutritional strategies need to be developed for newborns, particularly for premature infants where there is a high rate of morbidity and mortality and where specialized feeding regimens, including parenteral nutrition and tube feeding, are often employed. However, very little research pertaining to amino acid requirements and metabolism has been conducted in this population of neonates largely because of ethical and practical constraints. Premature neonates are often afflicted with a wide variety of other illnesses including heart, lung and intestinal disorders, making this population very heterogeneous (2,30). For ethical reasons, there are limitations to the dietary manipulations and experimental techniques that can be used in this vulnerable population and many clinically-acceptable research techniques may not be as sensitive as more invasive techniques that can be employed in animal models (2,30). In order to detect small, but important, differences in amino acid requirements and/or metabolism between treatment groups, a large number of research subjects would be required, and it is often difficult to recruit neonatal subjects. In most cases blood samples may only be taken as a part of the routine clinical monitoring of the neonate and not exclusively for research purposes; therefore, the sample volume available for analysis is often very small, and may limit the types of analyses that can be performed. For these reasons, it was necessary to develop an appropriate animal model for research relating to premature infants.

The neonatal piglet is an excellent experimental model for the human neonate, particularly the premature neonate (2,30,263). There are many similarities between the anatomy and physiology of human neonates and piglets including gastrointestinal maturation and development, maturation and function of the kidney, respiratory and circulatory system development (reviewed in 2). Neonatal piglets represent a readily available, homogeneous population, making them an economical and feasible choice for research. In addition, they are large enough at birth to perform delicate surgical procedures, such as catheter implantation, recover rapidly from surgery, and respond well to an experimental environment (2). Post-natal piglet growth is much more rapid than the growth of neonates, therefore small differences in metabolism may be detected over a relatively short experimental period.

Compared to humans, piglets spend relatively less time at the later stages of gestation (264) and because of this and the fact that they are a litter species, they are born less mature than full-term human neonates (2). The body composition of the newborn piglet more closely resembles that of a preterm infant than a full-term infant, with both the piglet and the preterm infant having relatively low fat reserves (265). Other similarities between preterm infants and piglets is that both have low thermoregulatory abilities and are susceptible to hyperglycemia (2). At birth, piglets are a very appropriate research model for the preterm infant; however, by 1 week of age it is likely that they have developed to the point that they are also an appropriate model for the term neonate.

The piglet and the infant are very similar with regards to amino acid and specifically arginine metabolism. The amino acid composition of the piglet and human bodies as well as the composition of sow's milk and human breast milk are very comparable (**Table 1.1**). In addition, both maternal milks are relatively deficient in arginine and high in proline (24), compared to the body tissue amino acid composition (**Table 1.1**), suggesting a reliance on *de novo* arginine synthesis in both species. Piglets and neonates are both sensitive to dietary arginine deficiency (10,34), and in both species the incidence of NEC is prevented by arginine supplementation (13,19). Until recently, much of the work relating to arginine metabolism was conducted in weaned or adult rodents which, as discussed extensively in the previous sections, differ greatly from neonates with regards to arginine metabolism; therefore, a suitable research model, such as the neonatal piglet, for the human neonate must be used in order to obtain clinically and nutritionally-relevant results.

The piglet as a model for the parenterally-fed human neonate has been well-validated (2,30,266). In addition, the enteral administration of an elemental diet with identical nutrient composition to a parenteral solution has also been validated in neonatal piglets (22,31). Therefore, not only will the results presented in this thesis contribute to the basic knowledge of amino acid metabolism in young piglets, but the results will also be relevant to the human neonatal population.

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2.0 RATIONALE AND OBJECTIVES

2.1 Scope of thesis

The aims of the research presented in this thesis were to examine the tissues sites of arginine synthesis in the neonatal piglet and to study the limitations in the metabolic pathway for endogenous arginine synthesis. Enterally-fed piglets were used in most studies, although a parenterally-fed piglet model was used to investigate the potential contribution of circulating precursors to endogenous arginine synthesis. Isotopes, either radioactive or stable, were infused either intragastrically, intraportally or intravenously to isolate the contributions of first-pass intestinal, first-pass hepatic and peripheral metabolism to whole-body arginine metabolism. The conversions between arginine and its various precursors were also examined using isotope infusions.

2.2 Rationale

Arginine intake from maternal milk is low relative to estimated daily requirements (1); therefore, there is a large reliance on endogenous arginine synthesis to meet metabolic needs. There is evidence that even the combination of dietary intake from milk or formula and endogenous synthesis may not be enough to promote optimal growth in piglets (2) and health in premature infants (3). However, despite the importance of arginine status and endogenous synthesis for neonates, very little research has been conducted *in vivo* to examine the tissue sites and limitations of endogenous arginine synthesis.

Based on research in parenterally and enterally-fed neonatal piglets, intact first-pass splanchnic metabolism is necessary for endogenous arginine synthesis (4); however, first-pass intestinal metabolism only accounted for 40-60% of whole-body arginine synthesis (5). Therefore, other sites of arginine synthesis must be present in neonatal piglets, and these sites require elucidation. In addition, although piglets receiving an arginine deficient diet did have a greater rate of endogenous arginine synthesis (5) than piglets receiving a generous arginine diet, there was an upper limit to endogenous arginine synthesis and the piglets receiving the arginine deficient diet still had the metabolic indications, such as elevated plasma ammonia and low plasma arginine concentration, of arginine deficiency. The portion of the arginine synthetic pathway limiting to endogenous arginine synthesis in neonatal piglets has not been identified.

By understanding the tissue sites of arginine synthesis and the limitations in the synthetic pathway, dietary strategies for infants and piglets may be optimized to either supply more arginine or to promote endogenous arginine synthesis, so that the metabolic requirements may be met and optimal growth and health can be promoted. This may be particularly important during times of metabolic stress, such as during weaning, or illness when the functions of one or more of the organs involved in arginine synthesis may be compromised.

2.3 Specific hypotheses and objectives

Hypothesis 1a: First-pass hepatic metabolism is not a site of endogenous arginine synthesis in enterally-fed neonatal piglets. **Hypothesis 1b**: Arginine synthesis during first-pass hepatic metabolism is not affected by arginine intake. **Hypothesis 1c**: Piglets receiving an arginine deficient diet will conserve arginine by reducing the rate of conversion of arginine to other amino acids.

The first study in this thesis examined the contribution of first-pass hepatic metabolism to whole-body arginine synthesis and determine whether or dietary arginine intake influenced the rate of first-pass hepatic arginine synthesis. A proline isotope was infused both intravenously and intraportally to isolate the role of first-pass hepatic metabolism on the conversion of proline to arginine (Hypothesis 1a), and piglets received either the deficient or generous arginine diets used in previous studies (5) (Hypothesis 1b). Proline to arginine conversion was used as a measure of endogenous arginine synthesis because proline is the major precursor *in vivo* for arginine synthesis (4,5). Arginine flux was measured with either [guanido-^{14}C]arginine, an isotope where the label was lost as urea when arginine is converted to urea, or [4,5-^{3}H]arginine, an isotope where the label remained with the ornithine moiety following urea formation, to assess the effect of arginine intake on label recycling and conversion of arginine to other metabolic products (Hypothesis 1c).

Hypothesis 2a: Parenterally-fed neonatal piglets have a lower rate of endogenous arginine synthesis from circulating precursors than enterally-fed piglets. **Hypothesis 2b**: The rate of endogenous arginine synthesis in parenterally-fed piglets is related to the degree of intestinal atrophy.

The rate of conversion of proline to arginine in parenterally-fed piglets receiving a low arginine diet was measured in the second study in this thesis, using the intravenous infusions of proline and arginine isotopes (Hypothesis 2a), and compared to the rate of arginine synthesis from circulating precursors in enterally-fed piglets (measured in Study 1). One group of piglets received a continuous infusion of glucagon-like peptide 2 (GLP-2) throughout the study to improve the parenteral-feeding induced intestinal atrophy, so that the effects of intestinal atrophy on the rate of arginine synthesis from circulating proline could be evaluated (Hypothesis 2b).

Hypothesis 3a: Citrulline is a more effective arginine precursor than either ornithine or proline in enterally-fed neonatal piglets. **Hypothesis 3b**: First-pass splanchnic metabolism extracts a substantial portion of dietary arginine intake and this extraction is not affected by whole-body arginine status.

Enterally-fed piglets were allocated to receive either the arginine-deficient diet, or the deficient arginine diet supplemented with equimolar amounts of proline, ornithine, citrulline or arginine. Whole-body arginine status was assessed using plasma ammonia, urea and amino acid concentrations and arginine flux. The principle behind this design was that piglets receiving a diet supplemented with an effective arginine precursor would have a similar whole-body arginine status to piglets receiving the arginine supplemented diet (Hypothesis 3a). In addition, the limitation for arginine synthesis in enterally-fed piglets would occur at a step in the arginine synthetic pathway prior to the formation of the effective arginine precursor(s). The extraction of dietary arginine by the splanchnic tissues, and the effects of whole-body arginine status on this extraction, was measured using the intravenous and intragastric infusion of an arginine isotope to measure arginine flux (Hypothesis 3b). An intragastric arginine flux significantly greater than the intravenous arginine flux, indicated a significant first-pass splanchnic extraction of dietary arginine.

Hypothesis 4a: Ornithine α-ketoglutarate is a more effective arginine precursor than ornithine-HCl in enterally-fed neonatal piglets. **Hypothesis 4b**: Ornithine metabolism is affected by ornithine intake. **Hypothesis 4c**: First-pass intestinal metabolism is an important site of ornithine metabolism.

In the fourth study presented in this thesis, piglets received the arginine deficient diet, or the arginine deficient diet supplemented with either ornithine, ornithine + α-ketoglutarate in a 2:1 molar ratio, or α-ketoglutarate and whole-body arginine status was assessed using the same criteria as Study 3 (Hypothesis 4a). An ornithine isotope was infused to measure the conversion of ornithine to other metabolic products, such as CO_2, arginine, proline and glutamate (Hypothesis 4b). The ornithine isotope was infused both intraportally and intragastrically so that the role of first-pass intestinal metabolism in ornithine metabolism could be elucidated (Hypothesis 4c).

Hypothesis 5a: Piglets receiving a deficient arginine diet will have greater rates of total arginine synthesis than piglets receiving a generous intake of arginine. Proline is the major precursor for endogenous arginine synthesis in enterally-fed neonatal piglets. **Hypothesis 5b**: Citrulline formation limits endogenous arginine synthesis from proline in enterally-fed piglets. **Hypothesis 5c**: Nitric oxide synthesis is lower in piglets receiving a deficient versus generous arginine diet.

In the final study of this thesis, the same deficient and generous arginine diets as the first study were used. An intragastric infusion of four stable isotopes: arginine, citrulline, ornithine and proline, each producing different isotopomers for the amino acids being studied, allowed for the measurement of the whole-body inter-conversions between these four amino acids (Hypothesis 5a). The relative importance of proline as an arginine precursor was assessed by comparing the rate of proline conversion to arginine with the rate of citrulline conversion to arginine, which was equivalent to the total rate of arginine synthesis (Hypothesis 5a). In addition to the major objectives of this study, the previously determined rates of whole-body arginine synthesis from proline (5) and the limiting portions of the proline to arginine metabolic pathway (Hypothesis 2) were reconfirmed using this stable isotope, multi-tracer methodology (Hypothesis 5b). The conversion of arginine to citrulline was used as a measure of nitric oxide synthesis, because for each mol of citrulline formed from arginine, there was also a mol of nitric oxide formed (Hypothesis 5c).

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3.0 ARGININE SYNTHESIS DOES NOT OCCUR DURING FIRST-PASS HEPATIC METABOLISM IN THE NEONATAL PIGLET[1]

3.1 Introduction

In week-old piglets it is proline and not glutamine/glutamate that is the major precursor for the carbon backbone required for arginine synthesis (1-3). Addition of proline to an arginine-free diet prevented severe hyperammonemia when neonatal piglets were enterally, but not parenterally, fed (1). These findings suggested that proline can only act as an arginine precursor when first-pass splanchnic metabolism is intact (1). Indeed, Bertolo et al. (4) showed that first-pass intestinal, and not hepatic or peripheral metabolism, was responsible for the conversion of proline to ornithine. These observations were presumably due to the primary localization of ornithine aminotransferase (OAT, EC number 2.6.1.13) to the small intestine (5). However, a subsequent study found that although whole-body arginine synthesis was one-fold greater in enterally-fed piglets receiving a deficient arginine diet, relative to piglets fed a generous arginine diet, first-pass intestinal metabolism was only necessary for between 42-63% of whole-body arginine synthesis and was not affected by arginine intake (2). Therefore, there must be sites other than first-pass intestinal metabolism involved in arginine synthesis in the neonatal piglet, and it is the synthesis at these sites that is regulated by arginine intake. One such site is first-pass hepatic metabolism, which is also bypassed during parenteral feeding.

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[1] A version of this chapter has been published. Urschel et al. 2005. *Am J Physiol Endocrinol Metab.* 288: E1244-E1251

The neonatal porcine liver has detectable activity of the two enzymes necessary to convert proline to ornithine, proline oxidase (EC number not assigned) and OAT (5,6). Because the piglet liver also has complete urea cycle enzymatic activity (7-9), the piglet liver has all the enzymes necessary to synthesize arginine from proline; however, whether this actually occurs and can release measurable quantities of arginine to the rest of the body has not been quantified. We hypothesized that first-pass hepatic metabolism in enterally-fed neonatal piglets could account for the difference between whole-body arginine synthesis and first-pass intestinal arginine synthesis. In the present study, our first goal was to examine arginine synthesis under dietary conditions previously shown to result in both basal and maximal rates of endogenous arginine synthesis (2).

Arginine synthesis in adult men (10) and rats (11) appears to be independent of arginine intake, and in men the metabolic response to an arginine-free diet is a decrease in the rate of ornithine oxidation (12). Neonatal piglets are able to increase arginine synthesis in response to arginine deficiency (2), although their ability to decrease arginine hydrolysis during arginine deficiency has not been examined. The portion of the plasma urea that is derived from dietary arginine is five times higher in piglets fed a generous arginine diet than in piglets fed a deficient arginine diet (2), suggesting that the hydrolysis of dietary arginine is decreased, as a conservation mechanism, during times of inadequate arginine intake. We hypothesized that if piglets are able to conserve arginine during times of deficiency, then this will result in a lower relative conversion of arginine to other urea cycle intermediates, such as ornithine and citrulline, when compared to piglets fed a generous amount of arginine.

The conversion of arginine to either citrulline or ornithine can only be measured with a label that is not removed during urea formation, such as [4,5-^{3}H]arginine. Because the label remains in the urea cycle, this results in an accumulation of the label in arginine as well as the other urea cycle intermediates. We hypothesized that the label recycling of the [4,5-^{3}H]arginine isotope would result in an underestimation of arginine flux and an overestimation of the conversion of arginine to urea cycle intermediates.

3.2 Materials and methods

3.2.1 *Animals and surgical procedures*

All procedures in this study were approved by the Faculty Animal Policy and Welfare Committee at the University of Alberta. Sixteen intact male Landrace/ Large White piglets (Hypor, Regina SK, Canada) (1.5 – 2.0 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant catheters.

Prior to surgery (d 0), piglets were given an intramuscular injection of ampicillin sodium (20 mg/kg; Novopharm, Toronto ON, Canada), and then pre-anaesthetized with an intramuscular injection of acepromazine maleate (0.22 mg/kg; Atravet; Wyeth Laboratories, Guelph ON, Canada) and atropine sulfate (0.05 mg/kg; MTC Pharmaceuticals, Cambridge ON, Canada). Piglets were then intubated, and anaesthesia was maintained throughout surgery using 1% halothane. Each piglet was implanted with gastric, jugular vein, femoral vein (TiCell Bioservices, Thornhill ON, Canada), and umbilical vein (0.040" ID x 0.085" OD silastic tubing; Dow Corning Corporation, Midland MI, USA) catheters using previously described aseptic techniques (13). A Stamm gastrostomy (14) was performed to implant the gastric feeding catheter. The femoral catheter used for blood sampling was inserted into the left femoral vein and then advanced to the inferior vena cava just caudal to the heart. The jugular catheter used for isotope infusion was inserted into the left jugular vein and then advanced to the superior vena cava just cranial to the heart (13). The portal catheter used for isotope infusion was implanted by introducing the catheter transperitoneally into the umbilical vein and then advancing it to the portal-hepatic junction (4). To ensure the patency of the umbilical vein catheter, a 0.9% saline solution was infused into this catheter at a rate of 1.2 $mL \cdot hr^{-1}$ throughout the entire trial.

3.2.2 *Post-surgical piglet care and housing*

Following surgery, piglets were given an intramuscular injection of the analgesic buprenorphine hydrochloride (0.03 mg/kg; Buprenex; Rekitt and Colman Pharmaceutical, Richmond VA, USA). Piglets were given additional intramuscular injections of buprenorphine hydrochloride 8 and 16 hours later. Ampicillin sodium was given intravenously into the jugular vein catheter (10 mg/kg), every twelve hours on d 1 and d 2 and gentamicin sulfate (2.5 mg/kg; Garasol; Schering-Plough Animal Health, Pointe-Claire PQ, Canada) was given intramuscularly on the mornings of d 1 and d 2. A topical antibacterial and antifungal creme (Hibitaine Veterinary Ointment; Wyeth Laboratories, Guelph ON, Canada) was used on all incision sites.

Piglets were housed individually in circular wire mesh cages, approximately 75 cm in diameter. These cages allowed for visual and audio contact between the piglets, and toys were provided for additional environmental enrichment. Lighting was on a 12-h light: dark schedule, and the room temperature was maintained between 21 – 27 °C, with localized supplemental heat provided by heat lamps.

3.2.3 *Diets*

A complete elemental diet, designed to meet the nutrient requirements of neonatal piglets (13), was continuously infused enterally via the gastric catheter throughout the experiment using pressure-sensitive infusion pumps (IVAC 560; IVAC, San Diego CA, USA). Piglets were fitted into cotton jackets that allowed them to be secured to a tether. The tether is part of a swivel-tether system (Alice King Chatham Medical Arts, Hawthorne CA, USA) that allows piglets to move freely while they are receiving a constant diet infusion, without the risk of catheters becoming tangled or occluded. The targeted nutrient intakes were 15 g amino acid/(kg·d), and 1.1 MJ metabolizable energy/(kg·d), with glucose and lipid (Intralipid 20%; Fresenius Kabi AB, Bad Homburg, Germany) each providing 50% of the non-protein energy intake. The amino acid composition of the complete diet was similar to that of an elemental total parenteral nutrition solution that is based on human milk protein (Vaminolact; Fresenius Kabi, Bad Homberg, Germany). The amino acid composition of the complete diet was similar to that provided in Shoveller et al. (15), with the following exceptions: L-arginine (4.41 g/L), glycine (1.36 g/L), L-phenylalanine (2.24 g/L), L-serine (1.78 g/L) and L-tyrosine (0.43 g/L). The base solution (amino acid mixture + 90 g/L glucose) was prepared and stored using previously described procedures (13). Just prior to diet infusion, a mixture of fat- and water-soluble vitamins (Multi-12/K_1 Pediatric; Sabex Inc., Boucherville PQ, Canada), minerals [supplied at 200% of the NRC (16) requirement], iron dextran (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and lipid were added to the base solution. The ratio of lipid to base solution was 1:5.

Immediately following surgery, the complete diet was infused parenterally via the jugular catheter at 50% of targeted intake until the morning of d 1. Diet infusion was then continued via the gastric catheter at 50% of targeted intake for 12 hours, 75% of targeted rate for 12 hours, and then at a full target rate [13.5 mL/(kg·h)] for the remainder of the trial. On the morning of d 3 piglets were randomly assigned to receive a diet containing either a generous [1.80 g/(kg·d) or 6.62 g/L in base solution; n = 8] or deficient [0.20 g/(kg·d) or 0.74 g/L in base solution; n = 8] concentration of arginine. These diets were chosen to represent diets that would result in minimum and maximum rates of arginine synthesis (2) without creating adverse metabolic effects (1). To ensure that the diets were isonitrogenous, the concentrations of alanine (generous arginine diet: 1.37 g/L; deficient arginine diet: 9.64 g/L) and glycine (generous arginine diet: 0.44 g/L; deficient arginine diet: 3.61 g/L) in the base solution were adjusted.

3.2.4 *Blood sampling*

Beginning on the morning of d 3, before the allocation to test diets, blood samples (2 mL) were collected every 24 hours until the end of the trial on the evening of d7. The daily blood samples were used for the determination of plasma ammonia and urea nitrogen levels, and the blood sample taken on the morning of d 7 was also used for the determination of plasma amino acid concentrations. As described under *constant tracer infusions*, additional blood samples were taken during tracer infusions on d 5, d 6 and d 7.

3.2.5 *Constant tracer infusions.*

On the morning of d 5, proline kinetics were determined by a primed [740 kBq (20 μCi)·kg^{-1}], constant [370 kBq (10 μCi)·kg^{-1}·h^{-1}] infusion of L-[U-^{14}C]proline (8.58 GBq/mmol; Amersham Biosciences, Baie d'Urfe PQ, Canada). Half of the piglets in each dietary treatment were given an intravenous infusion of the isotope via the jugular vein catheter, and the other piglets received an intraportal infusion via the umbilical vein catheter. The routes of infusion were crossed over on d 7, and piglets were infused with L-[U-^{14}C]proline via the route of infusion that they did not receive on d 5. The isotope was infused over a 7 h period, and blood (1 mL) was sampled at 0, 60, 120, 180, 240, 270, 300, 330, 360, 390 and 420 minutes. On d 7, an additional sample was taken one hour (–60 minutes) prior to the start of isotope infusion to correct for the background specific radioactivity (SA) of arginine, citrulline, glutamate, glutamine, hydroxyproline, ornithine and proline in the blood. During the intraportal infusions, the 1.2 mL·h^{-1} infusion of 0.9% saline was discontinued.

On d 6, whole-body arginine kinetics were determined using a primed [111 kBq (3 μCi)·kg^{-1}], constant [185 kBq (5 μCi)·kg^{-1}·h^{-1}] infusion of either L-[guanido-^{14}C]arginine (n=4 per treatment group) (1.9 GBq/mmol; NEN Life Science Products, Inc., Boston MA, USA) or L-[4,5-^{3}H]arginine (n=4 per treatment group) (1480 GBq/mmol; American Radiolabeled Chemicals, Inc., St. Louis MO, USA). Isotope was infused intravenously into the jugular catheter for 6.5 hours. Blood (1 mL) was sampled at –60, 0, 60, 120, 180, 240, 270, 300, 330, 360 and 390 minutes. The infusion doses and lengths for all infusions were based on previous experiments (2,4).

Following the isotope infusion on d 7, piglets were anaesthetized with halothane and were killed by the injection of 1000 mg sodium pentobarbital into the jugular vein catheter.

3.2.6 *Analytical procedures*

Plasma amino acid concentrations and the SA of arginine, citrulline, glutamate, glutamine, hydroxyproline, proline and ornithine were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (17,18). The internal standards norleucine and L-[4,5-^{3}H]leucine (1920 GBq/mmol; Amersham Pharmacia Biotech, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive derivatives were collected in 2 mL fractions, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter. The determination of the urea concentration and radioactivity was as previously described (4).

Plasma ammonia (Reference 200-02; Diagnostic Chemical Limited, Charlottetown PEI, Canada) and urea nitrogen (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA) concentrations were determined every 24 h during test diet infusion (d 3 – d 7) using spectrophotometric assays.

3.2.7 *Calculations*

The formulas and procedures that were used to calculate the plasma SAs of the post-column radioactive derivatives, the fractional net conversions of the precursor (either proline or arginine) to the product amino acids, the whole-body fluxes of the infused amino acids (proline and arginine), and the absolute conversions of proline to arginine ($Q_{\text{proline to arginine}}$) were as previously reported (2). The calculated flux values included the amino acids entering the plasma pool through all sources: dietary, *de novo* synthesis and protein breakdown. The first-pass hepatic contributions were calculated within piglet by subtracting the intravenous value from the intraportal value for both the proline fractional net conversions and the $Q_{\text{proline to arginine}}$. A similar approach has been previously used to isolate the effects of first-pass intestinal metabolism (2,4).

3.2.8 *Statistical analyses*

All data were analyzed using the mixed model of SAS Version 8.3 (SAS Institute, Cary NC, USA), and data were considered statistically significant if $P < 0.05$.

The dependent variables plasma ammonia and plasma urea nitrogen were analyzed using repeated measures analysis where the fixed effect was diet (generous or deficient arginine diet) and the random variables were piglet nested in diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis based on the Schwarz's Bayesian Criterion. When the effects were significant ($P < 0.05$), least squares means were separated using the pdiff option. We expected the plasma ammonia and urea nitrogen levels to be greater in piglets fed the deficient arginine diet (2); therefore a one-tailed t-test was used to compare values between treatment groups.

The results from the intravenous and intraportal proline infusions and the intravenous and intraportal $Q_{\text{proline to arginine}}$ were analyzed using a 2 x 2 factorial design with the diet, route of infusion and the interaction between diet and route of infusion as the fixed effects. Day of isotope infusion was tested as a covariate for the proline infusion data and was included in the model only when the effect was significant ($P < 0.05$). When there were no significant differences within a diet between the IV and IP values, the first-pass hepatic contribution was considered to be not significantly different from zero. 2 x 2 factorial analysis was also used to analyze the arginine flux data with the diet, isotope infused and the interaction between diet and isotope infused as the fixed effects. All other data were analyzed using diet as the fixed effect. For all statistical analyses, piglet nested in diet was used as the random term, and when the model $P < 0.05$, least squares means were separated using the pdiff option, and the two-tailed p-values were used to assess significance.

3.3 **Results**

3.3.1 *Piglet performance*

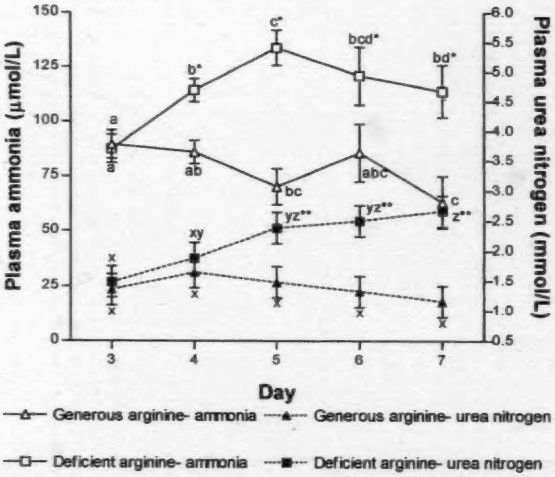
All piglets remained active throughout the entire trial. The umbilical catheter was not patent for 2 pigs per treatment group; therefore, these piglets received only the intravenous infusions. Thus, for all results involving the intraportal infusion, only 6 values per treatment group were available. Based on a priori power calculations, using our previous data (2), 6 values per treatment group is more than adequate to detect significant differences between the two diets for IP fluxes, fractional net conversions, $Q_{\text{proline to arginine}}$, as well as first-pass hepatic contributions.

There were no differences between the two treatment groups for piglet weight at initiation of test diet infusion (pooled mean = 2.08 kg, SE = 0.08 kg), rate of weight gain on test diet [pooled mean = 91 g/(kg·d), SE = 10 g/(kg·d)], and final piglet weight (pooled mean = 2.91 kg, SE = 0.16 kg).

3.3.2 *Plasma ammonia and urea nitrogen concentrations*

Both diet ($P = 0.0004$) and the interaction between day and diet ($P = 0.004$) had a significant effect on plasma ammonia concentrations (**Figure 3.1**). Piglets fed the deficient arginine diet had higher plasma ammonia concentrations ($P < 0.05$) than those piglets fed the generous arginine diet from the morning of d 4 onwards (**Figure 3.1**). Diet ($P < 0.0001$) and the interaction between day and diet ($P = 0.05$) also significantly impacted plasma urea nitrogen concentrations (**Figure 3.1**). From the morning of d 5 onwards, piglets fed the deficient arginine diet had higher plasma urea nitrogen concentrations ($P < 0.02$) than the piglets fed the generous arginine diet (**Figure 3.1**).

Figure 3.1 Mean plasma ammonia (μmol/L) and urea nitrogen (mmol/L) concentrations of piglets fed enteral diets with either a generous [1.80 g/(kg·d); n = 8] or deficient [0.20 g/(kg·d); n = 8] intake of arginine[1]



[1] Values are least squares means ± pooled SE. The unstructured variance-covariance matrix was chosen for the plasma ammonia analysis and the simple variance-covariance matrix was used for the plasma urea nitrogen analysis. The least squared means were separated using the pdiff option.

[abcd] For plasma ammonia concentrations within a diet, days not sharing a superscript are significantly different from each other ($P < 0.05$, one-tailed t-test).

[*] The piglets fed the deficient arginine diet had significantly ($P < 0.05$, one-tailed t-test) higher plasma ammonia concentrations than those fed the generous arginine diet from d 4- d 7.

[xyz] For plasma urea nitrogen concentrations within a diet, days not sharing a superscript are significantly different from each other ($P < 0.05$, one-tailed test).

[**] The piglets fed the deficient arginine diet had significantly ($P < 0.05$, one-tailed t-test) higher plasma urea nitrogen concentrations than those fed the generous arginine diet from d 5- d 7.

3.3.3 *Plasma amino acid concentrations*

On the morning of d 7, piglets fed the deficient arginine diet had significantly lower plasma concentrations of arginine and ornithine ($P < 0.01$; **Table 3.1**), and higher concentrations of asparagine and glutamine ($P < 0.05$, **Table 3.1**). Plasma threonine (deficient arginine diet: 470 μmol/L; generous arginine diet: 916 μmol/L; pooled SE: 139 μmol/L) and serine (deficient arginine diet: 511 μmol/L; generous arginine diet: 274 μmol/L; pooled SE: 26 μmol/L) concentrations were also affected by diet ($P < 0.05$). Alanine and glycine were present in higher concentrations in the arginine deficient diet than in the arginine generous diet to achieve equal nitrogen intake; therefore, the piglets fed the deficient diet had significantly greater plasma concentrations of these amino acids ($P < 0.0001$, data not shown). There were no other differences in plasma amino acid concentrations between the two treatment groups (data not shown).

3.2.4 *Amino acid fluxes*

Route of infusion and dietary treatment did not affect proline flux ($P > 0.05$, **Table 3.2**). The whole-body arginine fluxes determined using both the L-[guanido-^{14}C]arginine and the L-[4,5-^{3}H]arginine were significantly greater in the piglets fed the generous arginine diet ($P < 0.0001$, **Table 3.2**). However, the calculated fluxes using the L-[4,5-^{3}H]arginine isotope were significantly lower for both treatment groups ($P = 0.01$) when compared to the values determined using the L-[guanido-^{14}C]arginine isotope (**Table 3.2**).

Table 3.1 Plasma concentrations of amino acids involved in the urea cycle and arginine synthesis in piglets enterally-fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g/(kg·d)] diet for 5 days[1]

Amino acid	**Generous arginine diet** (n = 8)		**Deficient arginine diet** (n = 8)	**Pooled SE**	**P <**
		(μmol/L)			
Arginine	257		27	38	0.01
Aspartate	10		14	2	0.15
Asparagine	15		40	3	0.0001
Citrulline	66		86	8	0.15
Glutamate	143		144	17	NS[2]
Glutamine	103		347	33	0.05
Hydroxyproline	100		85	9	NS
Ornithine	170		39	15	0.0001
Proline	627		647	86	NS

[1] Data represent least squares means. Least squared means were separated using the pdiff option. [2] NS indicates the $P > 0.20$.

Table 3.2 Plasma proline and arginine fluxes [μmol/(kg·h)] of neonatal piglets enterally-fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g/(kg·d)] diet[1]

	Generous arginine diet	Deficient arginine diet	Pooled SE	P-value
Intraportal Proline Flux	*n = 6*	*n = 6*		
	381	323	50	NS
Intravenous Proline Flux	*n = 8*	*n = 8*		
	425	384	43	NS
Intravenous Arginine Flux	*n = 4*	*n = 4*		
(L-[guanido-^{14}C]arginine)	499	256	22	0.0001
Intravenous Arginine Flux	*n = 4*	*n = 4*		
(L-[4,5-^{3}H]arginine)	397[*]	166[*]	22	0.0001

[1] Data represent least squares means. Least squares means were separated using the pdiff option.

[*] $P < 0.05$ for within diet L-[4,5-^{3}H]arginine flux compared to L-[guanido-^{14}C]arginine flux.

3.2.5 *Proline conversion to other amino acids*

Day of isotope infusion was found to significantly ($P = 0.009$) affect only the conversion of proline to glutamate, and thus was only included as a covariate in the statistical model to analyze this data. Proline fractional net conversion to both arginine and ornithine was greater ($P < 0.0009$, **Table 3.3**) in piglets fed the deficient arginine diet for both the intraportal and intravenous infusions. The fractional net conversion of proline to glutamine was greater in the generous arginine group ($P = 0.01$, **Table 3.3**) for the intravenous infusion only. The only fractional net conversion affected by the route of isotope infusion was the proline to ornithine conversion in piglets fed the deficient arginine diet ($P = 0.008$, **Table 3.3**). Although we have calculated the first-pass hepatic fractional net conversions for all possible conversions (**Table 3.3**), with the exception of the conversion of proline to ornithine in piglets fed the deficient arginine diet, these values were not different from zero because there were no differences in the conversions between the intravenous and intraportal routes of infusion. Therefore, the difference in the first-pass hepatic fractional net conversions between diets was only significant for the proline to ornithine conversion ($P = 0.05$, **Table 3.3**).

3.2.6 *Arginine conversion to other amino acids*

For the L-[4,5-^{3}H]arginine isotope infusion, very little label was detected in glutamate, glutamine and hydroxyproline, leading to highly variable SA values. In addition, the proline SRA did not reach plateau in the 6.5 hour infusion for 4 of the 8 piglets. Therefore, plateaus in SA values for these amino acids were not obtained and fractional net conversions could not be calculated. Arginine conversion to citrulline,

Table 3. 3 Fractional net conversion of L-[U-^{14}C]proline to arginine, citrulline, glutamate, glutamine, hydroxyproline and ornithine in piglets fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g/(kg·d)] diet[1]

Conversion product	Generous arginine diet	Deficient arginine diet	Pooled SE	P <
		%		
Intraportal	*n* = 6	*n* = 6		
Arginine	3.3	15.8	1.4	0.0001
Citrulline	30.9	33.5	5.0	NS
Glutamate	4.1	3.6	0.5	NS
Glutamine	2.0	1.3	0.4	NS
Hydroxyproline	15.8	13.5	2.7	NS
Ornithine	10.6	18.2	1.4	0.01
Intravenous	*n* = 8	*n* = 8		
Arginine	3.1	18.4	1.2	0.0001
Citrulline	29.2	39.5	4.7	0.15
Glutamate	3.7	3.5	0.4	NS
Glutamine	2.7	1.3	0.3	0.05
Hydroxyproline	18.4	17.0	2.5	NS
Ornithine	10.9	23.3[†]	1.2	0.0001
First-pass hepatic[2]	*n* = 6	*n* = 6		
Arginine	0.2[‡]	-1.9[‡]	1.8	NS
Citrulline	2.9[‡]	-6.5[‡]	3.3	NS
Glutamate	0.8[‡]	-0.1[‡]	1.0	NS
Glutamine	-0.1[‡]	-0.1[‡]	0.5	NS
Hydroxyproline	-1.6[‡]	-3.8[‡]	2.1	NS
Ornithine	-0.5[‡]	-5.5[*]	1.6	0.05

[1] Data represent least squares means. Least squares means were separated using the pdiff option. Day of isotope infusion affected the fractional net conversion of proline to glutamate ($P = 0.009$), and therefore day was included in the statistical model as a covariate for glutamate only. [2] First-pass hepatic fractional net conversion was calculated within piglet by subtracting the intravenous fractional net conversion from the intraportal fractional net conversion.

[*] $P = 0.05$ compared to the generous arginine diet for the first-pass hepatic fractional net conversion of proline to ornithine

[†] $P = 0.008$ for intravenous compared to intraportal fractional net conversion of proline to ornithine in piglets fed the deficient arginine diet

[‡] Not different from zero, based on no significant difference between the intraportal and intravenous fractional net conversions

Table 3.4 Fractional net conversion of infused arginine isotope to other urea cycle intermediates in piglets fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g g/(kg·d)] diet[1]

Conversion product	**Generous Arginine diet**	**Deficient arginine diet**	**Pooled SEM**	**P <**
	%			
L-[guanido-^{14}C]arginine	*n*= 4	*n*= 4		
Urea	73	16	4	0.0001
L-[4,5-^{3}H]arginine	*n*= 4	*n*= 4		
Citrulline	57	8	2	0.0001
Ornithine	128	74	9	0.01

[1] Data represent least squares means. Least squares means were separated using the pdiff option.

ornithine and urea was significantly greater ($P < 0.0005$, **Table 3.4**) in piglets fed the generous arginine diet.

3.2.7 *Arginine synthesis from proline and the effect of first-pass hepatic metabolism*

There was a difference in the arginine flux calculated using the two arginine isotopes (**Table 3.2**). The ^{3}H on the 4,5 positions of the arginine are not lost with each turn of the urea cycle, unlike the ^{14}C on the guanido group. Therefore, the lower arginine flux calculated using L-[4,5-^{3}H]arginine is probably due to labeled arginine being recycled through the urea cycle. As a result, we used the mean arginine fluxes derived from the L-[guanido-^{14}C]arginine isotope infusion (**Table 3.2**) to calculate the $Q_{\text{proline to arginine}}$ (**Table 3.5**). This approach to calculating the $Q_{\text{proline to arginine}}$ is valid because of the significant difference in arginine fluxes between diets (**Table 3.2**, $P < 0.0001$) and confirms that the arginine flux is affected primarily by the level of dietary arginine. $Q_{\text{proline to arginine}}$ was greater in the piglets fed the deficient arginine diet both in the presence (intraportal; $P = 0.0002$) and absence (intravenous infusion; $P < 0.0001$) of first-pass hepatic metabolism (Table 5). However, first-pass hepatic metabolism did not contribute to the net whole-body $Q_{\text{proline to arginine}}$ ($P > 0.05$), regardless of the diet (**Table 3.5**).

Table 3.5 Synthesis of arginine from proline determined during intraportal and intravenous infusions of L-[U-^{14}C]proline in piglets enterally-fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g/(kg·d)] diet[1]

Arginine synthesis from proline	Generous Arginine diet	Deficient arginine diet	Pooled SE	P <
Intraportal	*n= 6*	*n= 6*		
μmol/(kg·h)	16.0	40.0	3.4	0.01
g/(kg·d)	0.07	0.17	0.01	0.01
Intravenous	*n= 8*	*n= 8*		
μmol/(kg·h)	15.5	47.1	3.5	0.0001
g/(kg·d)	0.06	0.20	0.01	0.0001
First-pass hepatic[2]	*n= 6*	*n= 6*		
μmol/(kg·h)	1.2*	-4.9*	4.8	NS
g/(kg·d)	0.01*	-0.02*	0.02	NS

[1] Data represent least squares means. Least squares means were separated using the pdiff option. [2] First-pass hepatic conversion of proline to arginine was calculated within piglet by subtracting the intravenous conversion from the intraportal conversion.

*Not different from zero, based on no significant difference between the intraportal and intravenous amounts of arginine synthesized from proline.

3.4 **Discussion**

3.4.1 *The role of first-pass hepatic metabolism in whole-body arginine synthesis*

To our knowledge, this is the first time that a multi-isotope, multi-site amino acid infusion has been used *in vivo* to separate hepatic metabolism from intestinal, peripheral and whole-body-metabolism in neonatal piglets. This research builds on similar approaches to fractional net conversion that have been successfully used to isolate the effects of first pass intestinal (2,4) and splanchnic (19) arginine metabolism. This study clearly demonstrated that the entire conversion of proline to arginine does not occur during first-pass hepatic metabolism, regardless of arginine intake, as evidenced by a lack of difference between the intraportal and intravenous fractional net conversions of proline to arginine or $Q_{\text{proline to arginine}}$ (Tables 3.3 and 3.5). Because the intraportally-infused proline tracer was exposed to first-pass metabolism by the liver, and the intravenously-infused proline tracer was not, the differences in the fractional net conversions and $Q_{\text{proline to arginine}}$ between these two routes can be attributed to first-pass hepatic metabolism.

Although the neonatal piglet intestine contains all of the enzymes necessary to convert both glutamine and proline to arginine (5,20,21), and *in vitro* studies have shown that in week-old piglet enterocytes, the net production of arginine from proline is less than that from glutamine (22), whole body studies in week-old piglets have clearly shown that it is proline that is the major precursor for arginine *in vivo* (1-3). A potential reason for the discrepancy between the *in vitro* and *in vivo* data is that glutamine is a source of the nitrogen moieties incorporated into arginine (22,23). These uses of glutamine/glutamate in arginine synthesis would not have been accounted for with the ^{14}C-glutamate used in the *in vivo* studies (2,3). Furthermore, Wilkinson et al. (2) found that when piglets are fed an arginine deficient diet, proline contributes to 27% of whole-body arginine flux while glutamate makes no contribution. Because the *in vivo* data suggest that in week-old piglets proline is the major source of the carbon backbone for arginine synthesis (2,3), the [U-^{14}C]proline used in the present study was a valid choice for a tracer to measure endogenous arginine synthesis.

Our finding that the liver does not release arginine to the rest of the body is in agreement with the adult mammalian data. Although urea cycle enzymes are abundant in the liver, hepatic release of arginine is low or absent in adult and growing rats (24-26), mice (27), and humans (28), even when fed an arginine-free diet (25,29). Adult mammalian hepatocytes have high arginase (EC number 3.5.3.1) activity (8,9,30) and there is tight channeling of urea cycle between the cytoplasmic urea cycle enzymes (31), presumably resulting in efficient ammonia detoxification to urea. Based on the present results, it appears that the metabolic priority of the urea cycle enzymes in the neonatal piglet liver is ammonia detoxification and not endogenous arginine synthesis.

We have previously used the same generous and deficient arginine diets to examine both whole-body and first-pass intestinal arginine synthesis (2). Based on the similar plasma ammonia (**Figure 3.1**), urea nitrogen (**Figure 3.1**) and plasma amino acid (**Table 3.1**) concentrations, and the intraportal fractional net conversions of proline to arginine (**Table 3.3**; (2)) between the two studies, the metabolic status of these piglets was similar between the present and previous (2) studies and therefore the results will be considered together to gain a more complete understanding of the sites of endogenous arginine synthesis in neonatal piglets.

Our previous study calculated the $Q_{\text{proline to arginine}}$ using an intragastrically infused arginine isotope to determine arginine flux (2), while the present study used an intravenously-infused arginine isotope. Both approaches are valid, as long as the distinction is made as to what the $Q_{\text{proline to arginine}}$ value represents. Flux calculated with the intragastrically-infused isotope includes the effects of first-pass splanchnic metabolism of the isotope; therefore, the resulting $Q_{\text{proline to arginine}}$ is the total endogenous arginine synthesis. $Q_{\text{proline to arginine}}$ calculated with the intravenously infused arginine isotope excludes the arginine that is synthesized and subsequently extracted by the splanchnic tissues during first-pass metabolism and thus represents net arginine synthesis. Arginine synthesis calculated using both the intravenous (**Table 3.2**) and intragastric (2) arginine flux values is presented in **Table 3.6**. First pass intestinal metabolism is necessary for ~60% and 40% of whole-body arginine synthesis when piglets are fed a generous or deficient diet, respectively (2). However, first-pass hepatic metabolism does not contribute to whole-body arginine synthesis (Tables 3.3 and 3.5). Therefore, the remainder of whole-body arginine synthesis must require sites not associated with first-pass splanchnic metabolism. In the present study, the synthesis of arginine from proline during peripheral metabolism was twofold higher in the piglets fed the deficient versus generous arginine diet (**Table 3.5**), suggesting that these are potential sites for the increased arginine synthesis during arginine deficiency.

Peripheral metabolism includes metabolism by other tissues, such as muscle and kidney, as well as second-pass splanchnic metabolism, which is the metabolism of arterially-derived substrate by the intestine and liver. Although Windmueller and Spaeth (26) observed a net release of citrulline from rat muscle, the muscle of neonatal piglets

Table 3.6 Sites of the fractional net conversion of proline to arginine and total and net arginine synthesis in piglets enterally-fed either a generous arginine [1.80 g/(kg·d)] or deficient arginine [0.20 g/(kg·d)] diet for 5 days

Site	Route of proline infusion[1]	Generous arginine diet		Deficient arginine diet	
		Total arginine synthesis[2]	Net arginine synthesis[3]	Total arginine synthesis[4]	Net arginine synthesis[5]
		g/(kg·d)	g/(kg·d)	g/(kg·d)	g/(kg·d)
Whole-body	IG	0.34[6]	0.17[6]	0.67[6]	0.29[6]
Whole body, excluding first-pass intestinal	IP	0.13[7]	0.06[7]	0.39[7]	0.17[7]
First-pass intestinal	IG-IP	0.21[8]	0.11[8]	0.28[8]	0.12[8]
Peripheral tissues/ second-pass splanchnic	IV	0.13[7]	0.06[7]	0.44[7]	0.19[7]
First-pass hepatic	IP-IV	0[9]	0[9]	0[9]	0[9]

[1] Refers to the route of the [U-^{14}C]proline infusion that was used to measure the fractional net conversion of proline to arginine for use in the calculation of arginine synthesis at each site; IG = intragastric, IP = intraportal, IV = intravenous. [2] Calculated using an intragastric arginine flux of 1011 µmol/(kg·h) (33). [3] Calculated using an IV arginine flux of 499 µmol/(kg·h) (**Table 3.2**, present study). [4] Calculated using an IG arginine flux of 590 µmol/(kg·h) (33). [5] Calculated using an IV arginine flux of 256 µmol/(kg·h) (**Table 3.2**, present study). [6] Fractional net conversion values used in the calculation were from Wilkinson et al. (33) (generous = 8%; deficient = 27%; pooled SE = 1%). [7] Fractional net conversion values used in the calculation were from the present study (**Table 3.3**). [8] Calculated by IG-IP. [9] Intravenous fractional net conversion was not significantly different from the intraportal conversion (**Table 3.3**); therefore, the first-pass hepatic conversion and arginine synthesis were negligible (Tables 3.3 and 3.5)

lacks proline oxidase activity (6) and has very low OAT activity (5) relative to the intestine. Because muscle does not appear to be able to convert proline to ornithine, the synthesis of arginine from proline by the muscle would not have been detected by the [U-^{14}C]proline infused in the present study. The kidney is crucial for arginine synthesis in adult mammals; however, it requires an exogenous source of citrulline (11), presumably due to the absence of the enzyme ornithine transcarbamoylase (OTC; EC number 2.1.3.3) (9). The piglet kidney also has the necessary enzymes to convert citrulline to arginine (21), as well as proline oxidase activity that is in the same order of magnitude as in the small intestine (6). However, the renal activity of OAT is approximately 40 times lower than in the jejunum (5) and in growing piglets there is no detectable renal OTC activity (9), which is likely preventing the conversion of substantial amounts of P5C to citrulline by the kidney. These findings do not preclude the possibility that the kidneys and muscles are involved in some portion of arginine synthesis in neonatal piglets, only that they are not likely capable of the entire proline to arginine synthetic pathway. The roles of these tissues in arginine synthesis in piglets require further investigation. Blood and nutrients from both the portal vein and the hepatic artery flow through the liver sinusoids (32); therefore, the effects of second-pass hepatic metabolism would be expected to be similar to those of first-pass hepatic metabolism. Thus second-pass hepatic metabolism cannot be a site of endogenous arginine synthesis (Tables 3.3 and 3.5).

Based on the results from the present study and of previous studies by our group (1,2,4,33), we hypothesize that second-pass intestinal metabolism is the site of increased arginine synthesis during arginine deficiency. The intestine has the enzymatic capability to convert proline to arginine (22), and the findings of Brunton et al. (1) suggest that intact first-pass splanchnic metabolism is necessary for the conversion of proline to arginine. However, the results from the present experiment and that by Wilkinson et al. (2) eliminate first-pass metabolism by both gut and liver as potential sites of increased arginine synthesis during arginine deficiency. The discrepancy in these observations may be explained by the importance of the enteral intake of nutrients in maintaining gut morphology (33) and splanchnic blood flow (34), both of which are negatively affected by parenteral feeding. The absence of healthy gut morphology and lower blood flow may have reduced the ability of the parenterally-fed gut to extract proline from arterial circulation to use in second-pass arginine synthesis (1). Although an arterial-venous difference study, in two-week old fasted piglets, concluded that there was no uptake of arterial proline by the jejunum (35), it is still possible that arterial proline may be extracted by the gut of the full-fed, week-old piglets used in the present study. The activities of the enzymes involved in endogenous arginine synthesis and the interconversions between arginine precursors and urea cycle intermediates both undergo changes between one and two weeks of age in neonatal porcine enterocytes (20-22). Furthermore, week-old piglet enterocytes synthesize a greater amount of arginine from proline than those from two-week old piglets (22), and thus arterial proline extraction may be necessary to sustain this synthesis. Research directly examining at the importance of second-pass intestinal metabolism in arginine synthesis is necessary.

3.4.2 *Splanchnic extraction of arginine*

The lower intravenous whole-body arginine fluxes observed in piglets fed the arginine deficient diet were expected (2,10), and were primarily due to the differences in arginine intake, which has also been observed in humans fed arginine-free versus arginine-supplemented diets (10,12,36). Approximately 80-95% of the difference in arginine fluxes between the two diets was accounted for by arginine intake, when arginine intake was corrected for first-pass splanchnic extraction using the approach of Castillo et al. (19). Using the intravenous arginine fluxes from the present study, in combination with the intragastric arginine fluxes of Wilkinson et al. (2) [arginine deficient: 590 μmol/(kg·h); arginine generous: 1011 μmol/(kg·h)), approximately 57% and 51% of the dietary arginine from the arginine deficient and generous diets, respectively, was extracted during first-pass splanchnic metabolism. First-pass intestinal extraction of dietary arginine in piglets is 40% (4); therefore, first-pass intestinal metabolism makes a larger contribution than first-pass hepatic extraction (11 - 17%) to total splanchnic extraction of dietary arginine. The conversions of arginine to urea during the IV infusion (**Table 3.4**) were similar to the results obtained from an intragastric infusion (generous: 68%; deficient: 14%; pooled SE = 3) (2), indicating that first-pass splanchnic metabolism is not a major site of dietary arginine hydrolysis in week-old piglets. Why piglet intestinal tissue extracts a substantial amount of arginine during first-pass metabolism is unknown. Other possible fates of the arginine extracted by the intestine include protein synthesis or the synthesis of metabolically active compounds such as polyamines or nitric oxide.

3.4.3 *Arginine conservation during dietary arginine deficiency*

Arginine fractional conversion to urea, ornithine and citrulline were 3.5, 1 and 10-fold greater, respectively, in piglets fed the generous versus deficient arginine diet (**Table**

3.4); therefore, arginine makes a smaller contribution to the fluxes of these metabolites in times of arginine deficiency. Although arginine is the immediate precursor to urea, circulating arginine only accounted for 16 and 73% of the whole-body urea flux (**Table 3.4**), when measured using the guanido-labeled arginine, for the deficient and generous arginine diets respectively. The remainder of the urea may be synthesized from intracellularly-derived arginine, especially during arginine deficiency. Urea derived from arginine produced intracellularly would not contain the label on the guanido carbon, and therefore would not be accounted for in our conversion calculation. Studies in isolated rat hepatocytes have found that arginine synthesized in the hepatocytes was the major source of arginine used for urea synthesis, due to a tight channeling between the cytoplasmic urea cycle enzymes (31). Furthermore, whole-body isotopic studies in adult men have shown that urea flux is substantially (~1.5 to 6 times) greater than arginine flux, which would not be the case if circulating arginine was the sole precursor for urea synthesis (37). The present results and those of others who have measured arginine conversion to urea using plasma kinetics (37) indicate that there may be intracellularly-derived arginine that does not equilibrate with the extracellular pool; therefore, these methods underestimate the whole-body arginine to urea conversion. Nevertheless, the results from the present study, using guanido-labeled arginine, clearly indicate that circulating arginine is a less important urea precursor when piglets are fed a deficient versus generous arginine diet (**Table 3.4**). This response is probably a conservation mechanism in response to the lower amount of circulating arginine (**Table 3.1**).

Neither ornithine nor citrulline is found in protein, nor was either provided in the diet, thus the only source of these amino acids in the extracellular pool was through endogenous formation. The present study demonstrates that during arginine deficiency less citrulline and ornithine were derived from arginine than when a generous amount of arginine is provided (**Table 3.4**). The lower conversion rates of arginine to both citrulline and ornithine suggest that the main metabolic response to arginine deficiency in piglets is decreased arginine hydrolysis. Compared to men fed a normal intake of arginine, men fed a deficient arginine diet had decreased arginine to ornithine conversion, as well as a decline in ornithine oxidation (12), while those consuming a therapeutic level of arginine had greater conversion of arginine to ornithine and a higher rate of ornithine oxidation (37). Therefore, when arginine deficient diets are fed, both adult humans and neonatal piglets conserve arginine by reducing its degradation.

In piglets fed the arginine deficient diet, there was a greater discrepancy between the two arginine flux values than in the piglets fed the generous arginine diet (35% vs. 20% lower for the [4,5-^{3}H]arginine versus the [guanido-^{14}C]arginine, **Table 3.2**). This indicates that arginine deficiency results in a greater amount of arginine recycling in piglets. This response agrees with our previous conclusion that arginine is conserved when it is limiting in the diet. The fractional net conversion of arginine to ornithine, calculated using the [4,5-^{3}H]arginine tracer, in the piglets fed the generous arginine diet was greater than 100% (**Table 3.4**), which further supports our hypothesis of label recycling. In a study in men fed an arginine enriched diet, and using an arginine isotope that is recycled, the portion of plasma ornithine flux derived from the arginine was determined to be 150% (37). The authors proposed that plasma arginine may be sequestered in a distinct intracellular pool where ornithine is synthesized and then released back into the plasma, resulting in an overestimation of whole-body arginine to ornithine conversion (37). Our data does not allow us to comment on their proposal of a distinct intracellular pool that sequesters ornithine; however, our data clearly indicates that recycling probably accounts for a substantial portion of the ornithine flux that they observed.

3.4.4 *Conclusion*

In conclusion, the neonatal porcine liver *in vivo* is not capable of synthesizing arginine from proline during first-pass metabolism, even when fed a limiting arginine diet. Whole body arginine synthesis is upregulated when a deficient arginine diet is fed; however, because first-pass intestinal arginine synthesis was not affected by arginine intake (2), extra-splanchnic tissues or second pass intestinal metabolism are likely responsible for this additional synthesis. In addition, piglets fed a deficient arginine diet also appear able to conserve circulating arginine by decreasing its conversion to other urea cycle intermediates. This study clearly demonstrates that, in neonatal piglets, whole body arginine synthesis is upregulated when arginine is deficient but not by first pass intestinal or hepatic metabolism. The site of increased arginine synthesis during arginine deficiency requires further investigation.

3.5 Literature Cited

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4.0 PARENTERALLY-FED NEONATAL PIGLETS HAVE AN EXTREMELY LOW RATE OF ENDOGENOUS ARGININE SYNTHESIS FROM CIRCULATING PROLINE[1]

4.1 Introduction

The primary metabolic uses for arginine include protein synthesis, ammonia detoxification to urea via the urea cycle, and the synthesis of creatine, nitric oxide and polyamines (1). Arginine synthesis and metabolism are well-described in adult and juvenile mammals (1,2); however, research in neonates has found that neonatal arginine metabolism is very different from adult arginine metabolism (3-5). Low plasma arginine concentrations are frequently seen in pre-term, as compared to term, neonates (5), and preterm infants that develop necrotizing enterocolitis (NEC) have lower plasma arginine concentrations than those infants that do not develop NEC (6). The provision of supplemental arginine to preterm infants reduced the incidence of NEC (7). Arginine conversion to nitric oxide is lower in infants during the acute phases of persistant pulmonary hypertension of the neonate (PPHN), as compared to during recovery from the acute episode (8), and the provision of a bolus dose of arginine during PPHN has been shown to improve infant oxygenation (9). Because of these critical roles of arginine, a complete knowledge of arginine metabolism in neonates is essential. However, because of ethical and practical constraints associated with experimentation in human neonates (10), a suitable animal model was needed to study arginine metabolism in neonates. The parenterally-fed neonatal piglet has been well-validated as an experimental model to study amino acid metabolism in parenterally-fed human neonates (10-13), and the majority of the research, both *in vitro* and *in vivo*, relating to arginine metabolism in neonates has been conducted in neonatal piglets (4,14-18).

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[1] A version of this chapter has been accepted for publication in *J Nutr* (December 19, 2007). A portion of this chapter has been accepted for publication in *Livestock Science*.

Recently, we have attempted to determine which tissues are important for arginine synthesis in neonatal piglets. Although arginine synthesis in adult mammals occurs via the intestinal-renal axis (19,20), the neonatal intestine contains all enzymes necessary for arginine synthesis (14,17) and we and others have shown that arginine synthesis is primarily intestinal in neonates (16,17,21,22). First-pass intestinal metabolism, which is the metabolism of dietary nutrients by the small intestine before they are released into the portal vein, accounted for 40-60% of whole-body endogenous arginine synthesis (4) and first-pass hepatic metabolism did not contribute to whole-body arginine synthesis (15). Therefore, the remaining 40 – 60% of arginine synthesis must be by either the peripheral tissues, such as the muscle or kidney, or via the intestinal metabolism of arterial substrates. We hypothesized that the intestinal metabolism of circulating precursors could make an important contribution to whole-body arginine synthesis.

Parenterally-fed piglets receiving an arginine-free diet that contained a generous amount of proline, the major arginine precursor in neonatal piglets (4,17), experienced a rapid onset of hyperammonemia (16), indicating an extremely diminished capacity for endogenous arginine synthesis. However, a direct measurement of arginine synthesis in parenterally-fed neonatal piglets has not been previously obtained. Parenteral feeding bypasses both first-pass intestinal and hepatic metabolism; therefore, parenterally-fed piglets would be expected to have a lower rate of endogenous arginine synthesis than enterally-fed piglets. Additionally, if the intestinal metabolism of arterial proline does make a contribution to whole-body arginine synthesis, then the decrease in intestinal blood flow (23) and the intestinal atrophy (24) that occur during parenteral feeding will likely result in a lower rate of arginine synthesis from circulating precursors in parenterally-fed neonates than in enterally-fed neonates.

Glucagon-like peptide 2 (GLP-2) is a 33 amino acid gut hormone that is released from the distal ileum in response to enteral feeding. Plasma GLP-2 concentrations are approximately 50% lower in parenterally than in enterally-fed piglets (25,26). Intravenous infusion of supraphysiological [~10 nmol/(kg·d)] amounts of GLP-2 has been shown to improve intestinal structure (25,26), increase rates of intestinal cell proliferation and protein synthesis (26), and prevent the decrease in portal vein blood flood (27) normally observed in parenterally-fed piglets. Therefore, we hypothesized that by improving intestinal structure and blood flow in parenterally-fed neonatal piglets, using a continuous GLP-2 infusion, there would also be an increase in the ability of the intestine to endogenously synthesize arginine from circulating precursors such as proline.

The objectives of the present study were: 1) to determine the rate of arginine synthesis in parenterally-fed piglets and 2) to determine how improving intestinal structure and increasing the intestinal mucosal mass, by using a continuous GLP-2 infusion, affects the use of circulating proline for arginine synthesis in parenterally-fed piglets.

4.2 **Materials and methods**

4.2.1 *Animals and surgical procedures*

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee for the University of Alberta. On d 0, ten intact male Landrace/ Large White/ Duroc piglets (Hypor, Regina SK, Canada) (1.5 – 2.0 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant a jugular vein catheter for feeding and treatment solution infusion and a femoral vein catheter for blood sampling. The surgical procedures, post-surgical injections and care, and piglet housing were as previously described (15).

4.2.2 *Experimental treatments*

Immediately following surgery and upon the initiation of intravenous diet infusion, piglets were allocated to one of two treatment groups: either +GLP-2 (n = 5) or +Sal (n = 5). Piglets in the +GLP-2 group were continuously infused, at a rate of 1 ml/(kg·h), with a 9 mg/ml saline solution that contained 1.8 μg/ml human GLP-2 (Bachem Bioscience Inc, King of Prussia PA) and 2.5 mg/ml bovine serum albumin (BSA, Sigma-Aldrich Canada, Oakville ON). Therefore, these piglets received approximately 10 nmol/(kg·d) GLP-2. A previous dose-response study in parenterally-fed piglets (26), evaluated the effectiveness of three rates of GLP-2 infusion [2.5, 5 and 10 nmol/(kg·d)], in improving intestinal morphology and increasing intestinal protein synthesis and cell proliferation relative to parenteral feeding alone, and found that a GLP-2 dose of 10 nmol/(kg·d) was the optimal dose to improve all of these intestinal parameters. The BSA was added as a carrier for the GLP-2 and to stabilize it in the infusion solution. Piglets in the +Sal group received a 1 ml/(kg·h) infusion of a 9 mg/ml saline solution with 2.5 mg/ml BSA. Both solutions were administered via the jugular vein catheter for the duration of the experiment.

4.2.3 *Diet infusion protocol*

Immediately following surgery, piglets received a complete elemental diet at 50% of targeted rate for 12 hours, followed by another 12 hours at 75% of targeted rate via the jugular vein catheter. By the morning of d 1, all piglets were being fed at the targeted rate of 13.5 ml/(kg·h). Piglets were weighed each morning and diet and treatment solution infusion rates were adjusted accordingly.

4.2.4 *Diets*

The complete elemental diet was designed to meet all nutrient requirements of piglets (28), and the targeted nutrient intakes were 15 g amino acid/(kg·d), and 1.1 MJ metabolizable energy/(kg·d), with glucose and lipid (Intralipid 20%; Fresenius Kabi AB, Bad Homburg, Germany) each providing 50% of the non-protein energy intake. The amino acid and glucose compositions of the complete diet base solution (29), and the procedures used to make the base solution (11), were as previously described. Prior to diet infusion, mixtures of fat- and water-soluble vitamins (Multi-12/K_1 Pediatric; Sabex Inc., Boucherville PQ, Canada), minerals (supplied at 200% of the NRC (28) requirement), iron dextran (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and lipid were added to the base solution. The ratio of lipid to base solution was 1:5.

On the morning of d 3, all piglets were assigned to a low arginine diet. A low arginine diet was used because we have previously shown that a deficient arginine intake results in the maximal stimulus for endogenous arginine synthesis (4), allowing us to more easily detect differences in arginine synthesis due to experimental treatment. The rate of arginine intake, of 0.60 g/(kg·d) (2.21 g/l arginine in base solution), was chosen because this level of intake represents approximately 50% of the estimated daily arginine use in neonatal piglets (1) but does not result in life-threatening hyperammonemia (21). The alanine (8.14 g/L) and glycine (2.34 g/L) contents of the base diet were modified to ensure that this diet was isonitrogenous to the complete diet.

4.2.5 *Blood sampling*

Beginning the morning of d 3, immediately before piglets began receiving the arginine deficient diet, blood samples (1.5 ml) were taken every 24 hours until the morning of d 7. Whole blood was collected into heparinized tubes and immediately centrifuged at 3000 rpm for 10 minutes, and the plasma layer was removed and frozen at -20°C until the time of analysis. Daily plasma samples were used for the measurement of ammonia and urea concentrations, and amino acid concentrations were also determined in the d 7 sample. Additional blood samples were taken on d 6 and 7 as described under *Constant tracer infusions*.

4.2.6 *Constant tracer infusions*

On d 6 arginine kinetics were measured in all piglets using a primed [111 kBq (3 μCi)/kg], constant [185 kBq (5 μCi)/(kg·h)] infusion of L-[guanido-^{14}C]arginine (2.11 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). The isotope was infused over 6 hours and blood was sampled at 0, 60, 120, 180, 240, 270, 300, 330 and 360 minutes.

Proline kinetics and the conversion of proline to arginine were measured in all piglets on d 7, using a 7 hour primed [740 kBq (20 μCi)/kg], constant [370 kBq (10 μCi)/(kg·h)] infusion of L-[U-^{14}C]proline (9.32 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). Blood was sampled at -60, -30, 0, 60, 120, 180, 240, 300, 330, 360, 390 and 420 minutes. We have successfully used these prime and constant infusion rates, for both the arginine and proline isotopes, in other studies using the neonatal piglet model (4,15). During both isotope infusions, the arginine deficient diet was continuously infused.

4.2.7 *Intestinal sampling*

At the end of the d 7 infusion, piglets were anaesthetized with 5% isoflurane (AErrane; Baxter, Mississauga ON) and the entire small intestine was removed posterior to the ligament of Treitz. Piglets were then euthanized with a 500 mg of pentobarbital sodium (Euthansol, 340 mg/mL; Schering Canada Inc., Pointe Claire PQ) injection into the femoral vein catheter. The extracted small intestine was rinsed with ice cold saline and divided in half, with the proximal half being designated as the jejunum and the distal half as the ileum. The jejunum was then divided in half again, and a section of exactly 20 cm was excised from the centre of this division. The mucosa from this 20 cm segment was scraped and the weight was recorded. A 5 cm segment immediately distal to the 20 cm segment was preserved in a 10% buffered formalin solution (Histoprep; Fisher Scientific, Ottawa ON) for later histological analysis.

4.2.8 *Analytical procedures*

Histology samples from the mid-jejunum were prepared and analyzed by a certified veterinary pathologist at the University of Alberta, using previously described procedures (24).

Plasma ammonia (Reference 200-02; Diagnostic Chemical Limited, Charlottetown PEI, Canada) and urea nitrogen (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA) concentrations were determined every 24 h during test diet infusion (d 3 – d 7) using spectrophotometric assays.

Plasma amino acid concentrations and the specific activities (SA) of arginine and proline, in the infusion plasma samples, were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (30,31). The internal standards norleucine and L-[U-^{14}C]leucine (10.81 GBq/mmol; American Radiolabeled Chemicals, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive proline and arginine derivatives were collected in 3 mL fractions, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter (Tri-Carb 4000 series, Canberra Packard, Canada).

4.2.9 *Calculations*

Plasma SAs of the post-column radioactive derivatives of amino acids were calculated as:

Plasma SA (Bq/mmol) = amino acid radioactivity (Bq/L) / amino acid concentration (μmol/L).

The d 7 infusion SA values for arginine were corrected for radioactive background by subtracting the average arginine SA of the pre-infusion (-60, -30 and 0 minute) samples.

Plateau SA values for each amino acid were verified as having a slope not different from zero. All plateaus had ≥ 3 time points. The plateau SA values were used in the fractional net conversion and the whole-body flux calculations outlined below.

The fractional net conversion of proline to arginine, during the L-[U-^{14}C]proline infusion, was calculated by:

Fractional net conversion (% of arginine flux) = ($SA_{\text{arginine at plateau}}$ / $SA_{\text{proline at plateau}}$) X 100.

Whole-body fluxes for the infused proline and arginine were calculated as:

Flux [μmol/(kg·h)] = constant infusion rate [Bq/(kg·h)] / $SRA_{\text{infused amino acid at plateau}}$ (Bq/μmol).

The calculated flux values included the amino acids entering the plasma pool through the diet, *de novo* synthesis and protein breakdown, or leaving the pool through protein synthesis, oxidation or conversion to other metabolites.

The absolute conversion of proline to arginine ($Q_{\text{proline to arginine}}$), which was a measure of whole-body arginine synthesis, was calculated using the following formula:

$Q_{\text{proline to arginine}}$ [μmol/(kg·h)] = Fractional net conversion$_{\text{proline to arginine}}$ (% of arginine flux) X arginine flux [μmol/(kg·h)].

4.2.10 *Statistical analysis*

Unless specifically noted, all data were analyzed using the mixed model of SAS Version 8.3 (SAS Institute, Cary NC, USA), and data were considered statistically significant if $P < 0.05$. Statistical trends were considered at $0.05 < P < 0.10$. When the fixed effects were significant ($P < 0.05$), least squares means were compared using the pdiff test.

The dependent variables plasma ammonia and plasma urea nitrogen were analyzed using repeated measures analysis where the fixed effect was diet and the random variables were piglet nested in diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion.

The remaining variables, including mucosal mass, plasma amino acid concentrations, arginine and proline fluxes, and $Q_{\text{proline to arginine}}$ were analyzed using diet as the fixed effect and piglet nested in diet as the random variable.

The relationships of the intestinal parameters (mucosal mass and villus height) to $Q_{\text{proline to arginine}}$ and plasma citrulline concentrations were analyzed using the regression and correlation procedures of SAS.

4.3 **Results**

4.3.1 *Piglet performance*

All piglets remained healthy and active for the duration of the trial. There were no significant differences between treatment groups for (mean ± pooled SE) initial body weight (1.77 ± 0.09 kg), body weight on d 3 (2.13 ± 0.14 kg), rate of weight gain from d 3 to d 7 [81 ± 16 g/(kg·d)], and final body weight (d 7) (2.79 ± 0.16 kg).

4.3.2 *Intestinal parameters*

Mucosal mass was greater in piglets receiving the GLP-2 infusion than in those receiving the control saline infusion ($P < 0.05$) (**Table 4.1**). Villus height was ~ 40% greater in the +GLP-2 piglets ($P < 0.001$), and there was a trend ($P = 0.05$) towards higher villus cell numbers in piglets in the +GLP-2 group (**Table 4.1**). There was no effect of treatment on either crypt depth or crypt cell number ($P > 0.10$) (**Table 4.1**). The villus:crypt ratio was also higher in the +GLP-2 versus the +Sal piglets ($P < 0.01$) (**Table 4.1**).

4.3.3 *Indicators of whole-body arginine status*

The d 7 concentrations of the plasma amino acids related to arginine synthesis and metabolism were particularly affected by the infusion of GLP-2 versus the saline control (**Table 4.2**). Although there was no effect of treatment on plasma arginine concentrations, plasma citrulline concentrations were significantly ($P < 0.05$) greater in the piglets in the +GLP-2 group (**Table 4.2**). There was a trend towards lower plasma

Table 4.1 Jejunal mass and morphology in parenterally-fed piglets receiving a low arginine diet and a continuous infusion of either saline or GLP-2[1]

Parameter	Treatment +GLP-2	 +Sal	Pooled SE
Jejunal mucosal mass [mg/(cm·kg)]	29	19	2
Villus height (μm)	689	479*	28
Villus cell number (# of cells per villus)	302	206†	29
Crypt depth (μm)	103	100	6
Crypt cell number (# of cells per crypt)	43	42	2
Villus:crypt ratio	6.8	4.8	0.4

[1] Values are least square means, n = 5. *Indicates a significant treatment effect ($P < 0.05$). †Indicates a trend towards a treatment effect ($0.05 < P < 0.10$).

Table 4.2 Day 7 plasma concentrations (μmol/L) of amino acids related to arginine metabolism in parenterally-fed piglets receiving a low arginine diet and a continuous infusion of either saline or GLP-2[1]

Amino acid	Treatment +GLP-2	 +Sal	Pooled SE
Arginine	31	34	8
Asparagine	21	28	5
Aspartate	27	43[†]	6
Citrulline	63	42[*]	6
Glutamate	126	208[†]	30
Glutamine	178	269[†]	33
Ornithine	24	16[†]	3
Proline	397	328	69

[1] Values are least square means, n = 5. [*] Indicates a significant treatment effect ($P < 0.05$).
[†] Indicates a trend towards a treatment effect ($0.05 < P < 0.10$).

aspartate (P = 0.08), glutamate (P = 0.09) and glutamine (P = 0.09) concentrations and higher plasma ornithine concentrations (P = 0.08) in piglets receiving the GLP-2 infusion (**Table 4.2**). With the exception of a trend (P = 0.08) for higher plasma glycine concentrations in piglets receiving the GLP-2 infusion (+GLP = 1510 μmol/L; +Sal= 961 μmol/L; pooled SE= 196 μmol/L), the plasma concentrations of the other amino acids were not affected by treatment (data not shown). The greater plasma concentrations of the two main intermediates in the arginine synthetic pathway, citrulline and ornithine, suggest an increase in the flux of substrates through this pathway. The lower plasma aspartate and glutamate concentrations in the +GLP-2 piglets may reflect their use for arginine synthesis. Plasma glutamine concentrations have been previously shown to be greater in piglets with a poor whole-body arginine status and a limitation in urea cycle function (4,15,29), or in piglets with receiving a diet deficient in an indispensable (32,33) amino acid, presumably due to its metabolic role as a nitrogen scavenger.

In previous studies (4,15,29), plasma ammonia concentrations have been used as an indicator of urea cycle function, with high plasma ammonia concentrations indicating an impairment in urea cycle function. Day (P = 0.01), but neither treatment (P = 0.94) nor the interaction between day and treatment (P = 0.13) had a significant effect on plasma ammonia concentrations (**Table 4.3**). In piglets receiving the GLP-2 infusion there was no effect of day on plasma ammonia concentrations (**Table 4.3**); however, piglets in the +Sal group had significantly higher plasma ammonia concentrations on d 6 and d 7 than on d 3 (**Table 4.3**). There was no effect of treatment (P = 0.54), day (P = 0.37), or the interaction between treatment and day (P = 0.62) on plasma urea concentrations (**Table 4.3**).

Table 4.3 Plasma ammonia (μmol/L) and urea (mmol/L) concentrations in parenterally-fed piglets receiving a low arginine diet and a continuous infusion of either saline or GLP-2[1]

	Plasma ammonia concentrations			Plasma urea concentrations		
Day	+GLP-2	+Sal	Pooled SE	+GLP-2	+Sal	Pooled SE
3	110	63^{a}	47	1.92	2.69	0.57
4	199	127^{ab}	47	2.17	1.71	0.40
5	123	139^{ab}	47	2.50	1.69	0.51
6	138	230^{b}	47	1.95	1.39	0.42
7	198	227^{b}	47	2.49	2.03	0.58

[1] Values are least square means, n = 5. Means in a column without a common letter differ, $P < 0.05$.

4.3.4 *Arginine and proline fluxes*

In addition to plasma amino acid, ammonia and urea concentrations, whole-body arginine flux was also used as an indicator of whole-body arginine status. Similar to the other indicators studied (**Tables 4.2** and **4.3**), GLP-2 infusion, in conjunction with the low arginine diet, did not have a significant effect on arginine flux (**Table 4.4**). Proline flux was also not different between two treatment groups (**Table 4.4**).

4.3.5 *Proline conversion to arginine*

Because proline, and not glutamate, is the major arginine precursor in neonatal piglets *in vivo* (4), the molar conversion of proline to arginine is a measure of endogenous arginine synthesis. GLP-2 infusion resulted in a significant increase in the fractional net conversion of proline to arginine ($P < 0.0001$) (**Table 4.4**) and in absolute arginine synthesis from proline ($P = 0.03$) (**Table 4.4**). Because there were no differences in either proline flux (**Table 4.4**) or plasma proline concentrations (**Table 4.2**) between the two groups, the size of the proline pool was assumed to be similar in both groups and therefore was not the cause of the observed differences in arginine synthesis from proline.

4.3.6 *Relationship between intestinal parameters and $Q_{\text{proline to arginine}}$ and plasma citrulline concentration*

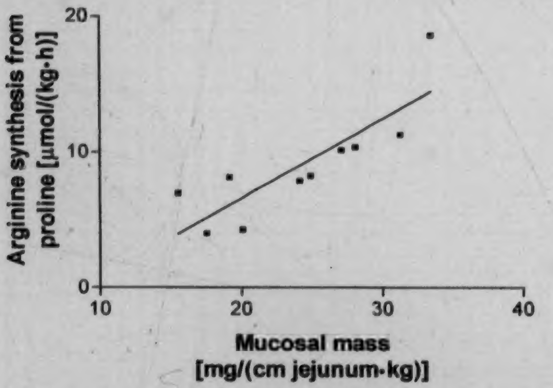
There was a strong, positive correlation ($r = 0.85$; $P = 0.002$) between mucosal mass and villus height. A strong, positive linear relationship ($R^2 = 0.72$; $P = 0.002$) was found between mucosal mass and arginine synthesis from proline (Figure 4.1). Villus height was also related to arginine synthesis from proline ($R^2 = 0.65$; $P = 0.004$).

Table 4.4 Plasma proline and arginine kinetics and absolute conversion of proline to arginine in parenterally-fed piglets receiving a low arginine diet and a continuous infusion of either saline or GLP-2[1]

	Treatment		
	+GLP-2	**+SAL**	**Pooled SE**
[U-^{14}C]proline infusion			
Proline flux [μmol/(kg·h)]	500	471	46
Fractional net conversion of proline to arginine (% of arginine flux)	11.3	6.0*	0.5
[guanido-^{14}C]arginine infusion			
Arginine flux [μmol/(kg·h)]	102	106	12
$Q_{\text{proline to arginine}}$[2]			
μmol/(kg·h)	11.7	6.3*	1.4
g/(kg·d)	0.05	0.03*	0.01

[1] Values are least square means, n = 5. [2] Rates of synthesis were calculated, within piglet, by multiplying the fractional net conversion of proline to arginine by arginine flux. *Indicates a significant treatment effect ($P < 0.05$).

Figure 4.1 Relationship between arginine synthesis from proline and mucosal mass in parenterally-fed piglets receiving either a GLP-2 (+GLP-2) or saline (+SAL) continuous infusion[1]



[1] From the regression analysis, there was a strong, positive, linear relationship between mucosal mass and arginine synthesis ($R^2 = 0.72$; $P = 0.02$).

Although there was no significant relationship between mucosal mass and plasma citrulline concentrations ($R^2 = 0.18$; $P = 0.22$), there was a significant linear relationship between villus height and plasma citrulline concentration ($R^2 = 0.51$; $P = 0.02$).

4.4 **Discussion**

4.4.1 *Arginine synthesis in parenterally-fed neonatal piglets*

To the best of our knowledge, this study is the first to directly measure arginine synthesis rates in parenterally-fed piglets. We have previously determined the basal and maximal rates of arginine synthesis in enterally-fed piglets receiving either a generous [1.80 g/(kg·d)] or deficient [0.20 g/(kg·d)] intake, respectively, of arginine (4,15). Enterally-fed piglets can synthesize up to 0.19 g/(kg·d) of arginine is synthesized from circulating precursors (15), which is ~3-5 fold greater than the rate of arginine synthesis in the parenterally-fed piglets of the present study [0.03-0.05 g/(kg·d); **Table 4.4**]. Therefore, parenterally-fed piglets synthesize substantially less arginine than enterally-fed piglets, when provided with a similar low daily arginine intake. However, the finding that parenterally-fed piglets are capable of even a small amount of endogenous arginine synthesis is a novel finding that is of both clinical and biochemical importance.

An important distinction between the previous enteral studies (4,15) and the present parenteral study is that the arginine content of the arginine deficient diet was lower [0.20 g/(kg·d) vs. 0.60 g/(kg·d)] in the enteral studies. We have previously shown that parenterally-fed piglets cannot be chronically maintained on less than 0.50 – 0.60 g/(kg·d) of arginine (21); therefore, we could not feed the same deficient arginine diet as in our previous enteral studies. However, compared to the enterally-fed piglets in the previous studies (4,15), the piglets in the present study had similar plasma ammonia, urea and arginine concentrations (**Tables 4.2** and **4.3**), indicating similar whole-body arginine status. Therefore, we do not believe that the greater dietary arginine allowance in the present study was the cause of the lower rate of arginine synthesis from circulating precursors in the parenterally versus enterally-fed piglets.

4.4.2 *Effects of GLP-2 infusion on intestinal parameters and arginine synthesis*

Mucosal mass and the jejunal histology parameters were examined in the present study to confirm that GLP-2 administration to parenterally-fed piglets was effective at minimizing the intestinal atrophy normally observed with parenteral feeding (25,34), as has been previously reported (25,26). Not only did piglets receiving the GLP-2 infusion have a greater mucosal mass and villus height than those receiving the saline infusion (**Table 4.1**), but the mid jejunum intestinal mass and villus height of the +GLP-2 and +Sal were similar to the values previously obtained from piglets of a comparable age (~10 days old) receiving enteral [~33 mg/(cm·kg); 534 μm] and parenteral diets [~20 mg/(cm·kg); 410 μm], respectively (24). Therefore, the desired differences in intestinal mass and structure were achieved with GLP-2 infusion, and we could then determine whether or not the improvements in the intestinal parameters were also associated with a greater rate of endogenous arginine synthesis.

Another indicator of intestinal function examined in the present study was plasma citrulline concentration. In adults with intestinal atrophy or short bowel syndrome, plasma citrulline concentration was highly correlated (r = 0.81 - 0.86) with both the degree of intestinal villus atrophy and short bowel length (35,36), and was deemed a powerful indicator of whether the intestinal failure was permanent or transient (36). These previous findings, in combination with the observation in rats that the small intestine is the only tissue that releases significant quantities of citrulline into circulation

(19), indicate that plasma citrulline concentration may be a valuable tool to assess intestinal health and metabolic function. In agreement with the other studies, piglets receiving the GLP-2, which had a greater mucosal mass (**Table 4.1**), had plasma citrulline concentrations that were 0.5-fold higher than in piglets receiving the saline infusion (Table 2). In addition, plasma citrulline concentration was linearly related to villus height ($R^2 = 0.51$), although not to mucosal mass ($R^2 = 0.18$). The lack of significant relationship between mucosal mass and plasma citrulline concentration may have been due to the relatively small sample size of this study. Therefore, similar to adults with intestinal dysfunction, plasma citrulline concentration also appears to be a reasonable indicator of villus height in parenterally-fed neonatal piglets; however, this relationship was not as strong as the relationship between the intestinal parameters and arginine synthesis from proline.

One of the primary objectives of the present study was to evaluate the importance of the intestinal utilization of circulating precursors for whole-body endogenous arginine synthesis. To address this objective we compared the rates of arginine synthesis in parenterally-fed piglets with either atrophied (+Sal) or non-atrophied (+GLP-2) small intestinal mucosa. Piglets receiving the GLP-2 had a 2-fold greater rate of endogenous arginine synthesis from proline (**Table 4.4**), as compared to those receiving the control saline infusion. Moreover, there were strong linear relationships between both villus height and mucosal mass (**Figure 4.1**) and the rate of arginine synthesis, providing strong, although indirect, evidence that improvements in intestinal parameters were associated with an increase in arginine synthesis. That the intestine was the most likely tissue to be responsible for the increase in arginine synthesis from proline due to GLP-2 infusion appears to be well-supported by the literature. The trophic effects of GLP-2 administration are believed to be primarily intestinal because the GLP-2 receptors are mainly located in the intestinal tissues (37,38). A previous study in parenterally-fed neonatal piglets, using the same infusion rate of GLP-2 as the present study, found an increased rate of protein synthesis in small intestine, but not in other tissues such as the stomach, liver, spleen and muscle (26). Furthermore, from that same study, mucosal thickness, but not muscularis thickness, was increased with increasing rates of GLP-2 infusion in parenterally-fed piglets (26). Therefore, the trophic effects of GLP-2 were largely limited to the small intestinal mucosa. In addition, the neonatal small intestine is the only known tissue, with the exception of the liver, to have all of the enzymes in the proline to arginine synthetic pathway (14,17); therefore, differences in arginine synthesis from circulating precursors between the +Sal and +GLP-2 groups can reasonably be largely attributed to differences in intestinal mucosal metabolism. In order to directly confirm that it was the intestinal metabolism of arterial precursors that was responsible for the increased conversion of proline to arginine, a more invasive arterio-venous difference study using isotopic tracers would need to be conducted. The relatively low arginine synthesis rate [0.03 g/(kg·d)] measured in the +Sal piglets represents the maximal total rate of arginine synthesis by either the atrophied small intestine, the synthesis by other peripheral tissues such as the kidney or muscle, or a combination of both of these factors.

4.4.3 *Effects of GLP-2 infusion on whole-body arginine status*

There were no significant differences between treatment groups for any of the indicators of whole-body arginine status (**Tables 4.2 - 4.4**), despite an almost 1-fold greater rate of arginine synthesis in the +GLP-2 piglets (**Table 4.4**), based on proline conversion to arginine. One possible reason for this apparent paradox is that compared to arginine intake, the difference in the rate of endogenous arginine synthesis between the two groups was small, only 3% of arginine intake. Therefore, although statistically significant, the higher rate of arginine synthesis from proline as a result of GLP-2 infusion appeared to be insufficient to be of physiological importance in improving the present measures of whole-body arginine status.

Our data (29) combined with that of Guan et al (27) show that the arginine synthesized by the small intestine may have been used locally, and was not released into general circulation, which is another potential reason why no differences in whole-body arginine status between the two treatment groups were observed. Using an arterio-venous difference method, Guan et al. (27) found that GLP-2 infusion in parenterally-fed piglets did not affect the rate of arginine release from the portal drained viscera. Furthermore, we have previously found that approximately 52% of enterally-infused arginine is extracted by the splanchnic region (29), regardless of whole-body arginine status. The metabolic fates of this extracted arginine are unconfirmed. However, the increased arginine synthesis by the small intestine of the +GLP-2 piglets was probably used to sustain the increased rate of intestinal protein synthesis (26), manifested as increased mucosal mass, or for nitric oxide synthesis, which is necessary for GLP-2 receptor function (27).

The low rates of arginine synthesis and the lack of differences in whole-body arginine status between treatment groups may also be related to the fact that there was a limitation in the metabolic pathway between proline and arginine that could not be overcome by GLP-2 administration. We have previously shown that there is a maximum amount of proline that can be used for arginine synthesis in enterally-fed piglets receiving an arginine deficient diet, due to a limitation in citrulline formation (29). Although plasma citrulline concentrations were higher in piglets receiving the GLP-2 infusion than in those receiving the saline infusion, the concentrations were still lower than those previously observed in enterally-fed piglets (~100 μmol/L) (4,15). Citrulline formation may have limited arginine synthesis in parenterally-fed piglets in the present study to a greater extent than in the enterally-fed piglets in previous studies (4,15).

A final potential reason for the very small effect of GLP-2 administration on arginine synthesis from proline, resulting in no effect on whole-body arginine flux, is that the low arginine intake may have limited the trophic effects of GLP-2. Arginine may be required for optimal GLP-2 effectiveness because the increase in intestinal blood flow associated with GLP-2 administration was nitric oxide dependent (27) and associated with an increase in endothelial nitric oxide synthase (EC number 1.14.13.39) mRNA and protein expression and protein phosphorylation (26,39). Because arginine is the precursor for nitric oxide synthesis (40), a limitation in dietary arginine could limit nitric oxide formation, and subsequently the effectiveness of GLP-2 administration on intestinal function. Although we did see improvements in villus height and mucosal mass with GLP-2 administration (**Table 4.1**), our intestinal improvements were not as dramatic as in the study by Burrin et al (26), which used the same rate of GLP-2 infusion, and this may have been due to the low arginine intake in the present study. We have previously shown that there is a trend for lower plasma nitric oxide concentrations in piglets receiving a deficient versus generous arginine diet (4); however, the effect of arginine intake on the rate of nitric oxide synthesis has not been measured in neonatal piglets. Future research is also necessary to determine whether arginine intake has an effect on the GLP-2 mediated intestinal response during parenteral feeding, and whether increasing the dose of GLP-2 administered results in an increase in the endogenous rate of arginine synthesis.

4.4.4 *Implications for neonatal nutrition*

The finding that there was only a very small increase in arginine synthesis from proline in parenterally-fed piglets, receiving a low arginine diet, is an important contribution to our current understanding of neonatal arginine metabolism and has important implications for neonatal nutrition. First, because the rate of arginine synthesis from circulating precursors in parenterally-fed piglets was only approximately 25% of the rate in enterally-fed piglets, it is critical that parenteral solutions provide enough arginine to satisfy the daily metabolic needs (1), as the contribution of endogenous synthesis is almost negligible. Arginine concentration in parenteral solutions is highly variable (4.7 – 12.3% of total amino acids by weight) (41), and we believe that some solutions do not provide enough arginine to maintain neonatal health (41).

Due to the direct relationship between mucosal mass and arginine synthesis, efforts should be taken to ensure that neonatal mucosal mass is maintained, specifically during parenteral feeding. The finding that arginine metabolism was influenced by mucosal mass in parenterally-fed neonates is likely applicable to the metabolism of other amino acids and this requires further investigation.

4.4.5 *Conclusion*

In closing, parenterally-fed piglets are capable of only a very small amount of endogenous arginine synthesis from circulating proline, approximately 25% of the amount that enterally-fed piglets can synthesize from circulating proline. Although endogenous arginine synthesis was increased slightly in response to GLP-2 administration, presumably due to improvements intestinal morphology, arginine status remained unchanged between the two treatment groups. Therefore, arginine must be considered an indispensable amino acid in parenterally-fed neonates.

4.5 Literature cited

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5.0 CITRULLINE IS AN EFFECTIVE ARGININE PRECURSOR IN ENTERALLY-FED NEONATAL PIGLETS[1]

5.1 Introduction

Arginine metabolism in neonatal piglets is an important area of current research, not only because of the various metabolic uses of arginine (1), but because arginine intake has been shown to be a limiting factor for suckling piglet growth (2), and reduced circulating arginine concentrations and a lower whole-body arginine flux have been associated with human neonatal pathologies such as necrotizing enterocolitis (3) and persistent pulmonary hypertension of the neonate (4), respectively. Despite the metabolic importance of arginine, arginine intake from sow's milk only accounts for ~ 40% of daily arginine use (5), and therefore endogenous arginine synthesis is critical for maintaining arginine homeostasis (6).

The arginine synthetic pathway involves the conversion of precursor amino acids, either proline, glutamine or glutamate to pyrroline-5-carboxylate (P5C), ornithine, citrulline and finally arginine (see (1) for a detailed figure). Sow's milk does contain substantial amounts of proline, glutamine and glutamate (7,8), and trace amounts of ornithine and citrulline (8). We have previously shown that proline must be the major dietary source of the five carbon backbone of arginine in neonatal piglets (9-11). However, piglets fed a proline-containing, arginine deficient diet still do not maintain optimum arginine status (11,12). These data indicate that although proline is the major precursor of arginine, proline supplementation alone cannot optimize arginine status in

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[1] A version of this chapter has been published. Urschel et al. 2006. *J Nutr.* 136: 1806-1813.

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neonatal piglets. Therefore, there is an upper capacity for endogenous arginine synthesis in neonatal piglets, and some portion of the arginine synthetic pathway is limiting.

The first objective of the present study was to determine the most effective arginine precursor as a means to identify which step of the arginine synthetic pathway may be limiting in enterally-fed, week-old piglets. Although previous studies in dogs (13), rats (14), cats (15), and growing pigs (16) have shown that citrulline is a better arginine precursor than ornithine, there are many interspecies and developmental differences in arginine metabolism (5,17,18). To add to this data, we compared arginine precursors in week-old piglets and in addition to studying the traditional indicators of whole-body arginine status, namely plasma ammonia, urea and amino acid concentrations, we also used isotopes to quantify differences in whole-body arginine flux and determined the extent to which each arginine precursor would spare the use of proline for arginine synthesis. Piglets were given a deficient arginine diet (basal), ,or the basal diet supplemented with equimolar amounts of either proline (+Pro), ornithine (+Orn), citrulline (+Cit) or arginine (+Arg). We have previously shown that the amount of arginine provided in the +Arg diet exceeded the piglet's daily arginine requirement (5,11). Therefore, if any proline, ornithine or citrulline was converted to arginine efficiently, then the piglets receiving the +Pro, +Orn and +Cit diets, respectively, should have a similar arginine status to piglets receiving the +Arg diet.

We have previously estimated that 51-57% of arginine is extracted during first-pass splanchnic metabolism (12) in piglets receiving arginine deficient or generous diets. However, this estimate was based on data from two separate studies (11,12), and thus we could not statistically analyze the results to determine whether dietary treatment had an effect on dietary arginine extraction. Castillo et al (19) found that 38% of dietary arginine was extracted during first-pass splanchnic metabolism in adult men fed an arginine rich diet, but did not investigate the effect of dietary arginine intake on this extraction. Arginine is critical for numerous metabolic functions in both neonates and adult mammals (5,20); therefore, it is important to know how much dietary arginine enters general circulation under different dietary circumstances. The second objective of this study was to determine if arginine intake and whole-body arginine status had a significant effect on the first-pass splanchnic extraction of dietary arginine.

5.2 Materials and methods

5.2.1 *Animals and surgical procedures*

All procedures in this study were approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta. Twenty-five intact male Landrace/ Large White piglets (Hypor, Regina SK, Canada) (1.5 – 2.0 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant a gastric catheter for diet and isotope infusion, a jugular vein catheter for isotope infusion, and a femoral vein catheter for blood sampling. The surgical procedures, post-surgical injections and care, and piglet housing were as previously described (12).

5.2.2 *Diets*

A complete elemental diet, designed to meet the nutrient requirements of neonatal piglets (12), was continuously infused enterally via the gastric catheter throughout the experiment using pressure-sensitive infusion pumps (IVAC 560; IVAC, San Diego CA, USA). The targeted nutrient intakes were 15 g amino acid/(kg·d), and 1.1 MJ metabolizable energy/(kg·d), with glucose and lipid (Intralipid 20%; Fresenius Kabi AB, Bad Homburg, Germany) each providing 50% of the non-protein energy intake. The amino acid composition of the complete diet was based on that of an elemental total parenteral nutrition solution that is based on human milk protein (Vaminolact; Fresenius Kabi, Bad Homberg, Germany), and the amino acid composition of the base solution is provided in **Table 5.1**. The base solution was prepared and stored using procedures similar to those previously described (21). Just prior to diet infusion, mixtures of fat- and water-soluble vitamins (Multi-12/K_1 Pediatric; Sabex Inc., Boucherville PQ, Canada), minerals (supplied at 200% of the NRC (22) requirement), iron dextran (Ferroforte; Bimeda-MTC, Cambridge ON, Canada) and lipid were added to the base solution. The ratio of lipid to base solution was 1:5.

Immediately following surgery, the complete diet was infused parenterally via the jugular vein catheter at 50% of targeted intake until the morning of d 1. Diet infusion was then infused enterally at 50% of targeted intake for 12 hours, enterally at 75% of targeted rate for 12 hours, and then enterally at full target rate [13.5 mL/(kg·h)] for the remainder of the trial. Therefore, all piglets reached full target dietary administration by the morning of d 2. Piglets were weighed every morning for the duration of the trial, and diet infusion rates were adjusted accordingly.

On the morning of d 3 piglets were randomly assigned to one of five test diets (**Table 5.1**): an arginine-deficient basal diet, or the basal diet supplemented with an equimolar amount of either proline (+Pro), ornithine (+Orn), citrulline (+Cit), or arginine (+Arg). To ensure that the diets were isonitrogenous, the concentrations of alanine and glycine were adjusted from the base solution concentrations (**Table 5.1**).

5.2.3 *Blood sampling*

Beginning on the morning of d 3, be[illegible]e allocation to test diets, blood samples (2 mL) were collected every 24 hours unti[illegible]d of the trial on the evening of d 7. The daily blood samples were used for the determination of plasma ammonia and urea

Table 5.1 Amino acid composition of the base diet solution for the complete diet, fed until the morning of d 3, and the test diets, fed from the morning of d 3 until the end of the trial[1,2]

Amino acid	Complete	Test diets				
		Basal	+Pro	+Orn	+Cit	+Arg
L-alanine	5.88 (66.0)	9.64 (108.2)	8.14 (91.3)	6.63 (74.4)	4.37 (49.0)	1.37 (15.4)
L-arginine[3]	4.41 (25.3)	0.74 (4.2)	0.74 (4.2)	0.74 (4.2)	0.74 (4.2)	6.62 (38.0)
L-aspartate	3.35 (25.1)	3.35 (25.1)	3.35 (25.1)	3.35 (25.1)	3.35 (25.1)	3.35 (25.1)
L-cysteine	0.81 (6.7)	0.81 (6.7)	0.81 (6.7)	0.81 (6.7)	0.81 (6.7)	0.81 (6.7)
L-glutamate	5.81 (39.5)	5.81 (39.5)	5.81 (39.5)	5.81 (39.5)	5.81 (39.5)	5.81 (39.5)
Glycine	0.44 (5.9)	3.61 (48.1)	2.34 (31.2)	1.07 (14.3)	0.44 (5.9)	0.44 (5.9)
L-histidine	1.72 (11.1)	1.72 (11.1)	1.72 (11.1)	1.72 (11.1)	1.72 (11.1)	1.72 (11.1)
L-isoleucine	2.54 (19.3)	2.54 (19.3)	2.54 (19.3)	2.54 (19.3)	2.54 (19.3)	2.54 (19.3)
L-leucine	5.77 (44.0)	5.77 (44.0)	5.77 (44.0)	5.77 (44.0)	5.77 (44.0)	5.77 (44.0)
L-lysine[4]	4.59 (31.4)	4.59 (31.4)	4.59 (31.4)	4.59 (31.4)	4.59 (31.4)	4.59 (31.4)
L-methionine	1.07 (7.2)	1.07 (7.2)	1.07 (7.2)	1.07 (7.2)	1.07 (7.2)	1.07 (7.2)
L-phenylalanine	2.24 (13.6)	2.24 (13.6)	2.24 (13.6)	2.24 (13.6)	2.24 (13.6)	2.24 (13.6)
L-proline[5]	4.60 (39.9)	4.60 (39.9)	8.49 (73.7)	4.60 (39.9)	4.60 (39.9)	4.60 (39.9)
L-serine	1.78 (17.0)	1.78 (17.0)	1.78 (17.0)	1.78 (17.0)	1.78 (17.0)	1.78 (17.0)
Taurine	0.26 (2.1)	0.26 (2.1)	0.26 (2.1)	0.26 (2.1)	0.26 (2.1)	0.26 (2.1)
L-threonine	2.94 (24.7)	2.94 (24.7)	2.94 (24.7)	2.94 (24.7)	2.94 (24.7)	2.94 (24.7)
L-tryptophan	1.18 (5.8)	1.18 (5.8)	1.18 (5.8)	1.18 (5.8)	1.18 (5.8)	1.18 (5.8)
L-tyrosine	0.43 (2.4)	0.43 (2.4)	0.43 (2.4)	0.43 (2.4)	0.43 (2.4)	0.43 (2.4)
L-valine	2.94 (25.1)	2.94 (25.1)	2.94 (25.1)	2.94 (25.1)	2.94 (25.1)	2.94 (25.1)
L-citrulline[6]	0	0	0	0	5.92 (33.8)	0
L-ornithine[7,8]	0	0	0	4.47 (33.8)	0	0
glycyl-tyrosine[9]	1.43 (5.6)	1.43 (5.6)	1.43 (5.6)	1.43 (5.6)	1.43 (5.6)	1.43 (5.6)

[1]Values in the table are amino acid concentrations in g/L (mmol/L). [2]This solution also contained 90 g/L (454 mmol/L) of dextrose. [3]Piglets in the +Arg group received 1.80 g/(kg·d) of arginine; all other treatment groups received 0.20 g/(kg·d) arginine. [4] L-lysine was added as an equimolar amount (5.74 g/L) of lysine-HCl. [5] Piglets in the +Pro group

received 2.31 g/(kg·d) of proline; all other treatment groups received 1.25 g/(kg·d) proline. [6]Piglets in the +Cit group received 1.61 g/(kg·d) of citrulline. [7]L-ornithine was added as an equimolar amount (5.70 g/L) of ornit[illegible]e-HCl. [8]Piglets in the +Orn group received 1.55 g/(kg·d) of ornithine. [9]Glycyl-tyrosine supplied 0.45 g/L (5.6 mmol/L) of glycine and 1.09 g/L (5.6 mmol/L) of tyrosine nitrogen concentrations, and the blood sample taken on the morning of d 7 was also used for the determination of plasma amino acid concentrations. As described under *constant tracer infusions*, additional blood samples were taken during tracer infusions on d 5, d 6 and d 7.

5.2.4 *Constant tracer infusions*

On the morning of d 5, arginine kinetics were determined by a primed [111 kBq (3 μCi)/kg], constant [185 kBq (5 μCi)/(kg·h)] infusion of L-[guanido-^{14}C]arginine (2.04 GBq/mmol; American Radiolabeled Chemicals, Saint Louis MO, USA; and 2.11 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). Half of the piglets in each dietary treatment were given an intravenous infusion of the isotope via the jugular vein catheter, and the other piglets received an intragastric infusion via the gastric catheter. On d 7, piglets were infused with L-[guanido-^{14}C]arginine via the route of infusion that they did not receive on d 5. The isotope was infused over a 5.5 h period, and blood (1 mL) was sampled at 0, 60, 120, 180, 210, 240, 270, 300, and 330 minutes. On d 7, additional samples were taken one hour (–60 minutes) and 30 minutes (–30 minutes) prior to the start of isotope infusion to correct for the background specific activity (SA) of arginine in the blood. Infusing the arginine isotope by both the intravenous and intragastric routes enabled us to calculate the first-pass splanchnic extraction of arginine.

On the morning of d 6, proline kinetics and the conversion of proline to arginine were determined by a primed [740 kBq (20 μCi)/kg], constant [370 kBq (10 μCi) /(kg·h)] infusion of L-[U-^{14}C]proline (8.58 GBq/mmol; Amersham Biosciences, Baie d'Urfe PQ, Canada). Isotope was infused intragastrically for 8 hours. Blood (1 mL) was sampled at

–60, -30, 0, 60, 120, 180, 240, 300, 330, 360, 390, 420, 450 and 480 minutes. The infusion doses and periods for all infusions were based on previous experiments (11,12,23). Diets were infused continuously throughout all isotope infusions.

Following the isotope infusion on d 7, piglets were anaesthetized with halothane and were killed by the injection of 1000 mg sodium pentobarbital into the jugular vein catheter.

5.2.5 *Analytical procedures*

Plasma amino acid concentrations and the SA of arginine and proline were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (24,25). The internal standards norleucine and L-[U-^{14}C]leucine (10.81 GBq/mmol; American Radiolabeled Chemicals, St. Louis MO, USA) were added to each 300 μL plasma sample. Post-column radioactive derivatives were collected in 3 mL fractions, 14 mL of scintillant (Biodegradable Counting Scintillant; Amersham Canada, Ltd., Oakville ON, Canada) was added, and samples were counted on a scintillation counter (Tri-Carb 4000 series, Canberra Packard, Canada). For urea, the plasma concentration for each of the infusion samples were measured using a spectrophotometric assay (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA), and the associated radioactivity of each sample was measured by collecting the underivatized urea peak during HPLC analysis (23). The urea peak elution time was verified using a radioactive urea standard.

Plasma ammonia (Reference 200-02; Diagnostic Chemical Limited, Charlottetown PEI, Canada) and urea nitrogen concentrations were determined every 24 h during test diet infusion (d 3 – d 7) using spectrophotometric assays.

5.2.6 *Calculations*

Plasma SAs of the post-column radioactive derivatives of both amino acids and urea were calculated as:

Plasma SA (Bq/mmol) = amino acid radioactivity (Bq/L) / amino acid concentration (mmol/L).

The d 7 infusion SA values for arginine and urea were corrected for radioactive background by subtracting the average arginine SA of the -60, -30 and 0 minute samples. Plateau SA values for each amino acid were verified as having a slope not different from zero. All plateaus had ≥ 3 time points. The plateau SA values were used in the fractional net conversion and the whole-body flux calculations outlined below.

Fractional net conversion is the overall contribution that a precursor amino acid makes to the product amino acid flux. Fractional net conversions of the precursor (either proline or arginine) to product metabolite (either arginine or urea) were calculated by:

Fractional net conversion (% of product amino acid flux) = $(SA_{\text{product amino acid (arginine or urea) at pleateau}} / SA_{\text{precursor amino acid (proline or arginine) at plateau}}) \times 100$.

Whole-body fluxes for the intragastrically-infused proline and the intravenously and intragastrically-infused arginine were calculated as:

Flux [μmol/(kg·h)] = constant infusion rate [(Bq/(kg·h)] / $SA_{\text{infused amino acid at plateau}}$ (Bq/μmol).

The calculated flux values included the amino acids entering the plasma pool through the diet, *de novo* synthesis and protein breakdown, or leaving the pool through protein synthesis, oxidation or conversion to other metabolites. The intragastric fluxes were influenced by both first-splanchnic metabolism and the metabolism by other peripheral tissues (such as muscle, kidney, intestinal metabolism of arterial substrates, lung), while intravenous fluxes only included the effects of metabolism by peripheral tissues.

The absolute conversion of proline to arginine ($Q_{\text{proline to arginine}}$) was calculated using the the intragastric arginine flux values using the following formula, and was used as a measure of arginine synthesis:

$Q_{\text{proline to arginine}}$ [μmol/(kg·h)] = Fractional net conversion$_{\text{proline to arginine}}$ (% of arginine flux) X intragastric arginine flux [μmol/(kg·h)].

First-pass splanchnic arginine to urea conversion was calculated within piglet by subtracting the iv from the ig value and the first-pass splanchnic extraction of arginine was calculated within piglet using the approach of Castillo et al. (19) which is as follows:

First-pass splanchnic extraction (%) = 100 – (arginine flux$_{\text{intravenous}}$ [μmol/(kg·h)] / arginine flux$_{\text{intragastric}}$ [μmol/(kg·h)]).

5.2.7 *Statistical analyses*

Unless otherwise stated, all data were analyzed using the mixed model of SAS Version 8.3 (SAS Institute, Cary NC, USA), and data were considered statistically significant if $P < 0.05$.

The dependent variables plasma ammonia and plasma urea nitrogen were analyzed using repeated measures analysis where the fixed effect was diet and the random variables were piglet nested in diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis based on the Schwarz's Bayesian Criterion. When the effects were significant ($P < 0.05$), pre-planned comparisons of least squares means were made using the pdiff test, which is the two-tailed pairwise comparison test used by the mixed procedure.

The results from the intravenous and intragastric arginine infusions were analyzed using a 5 x 2 factorial design with the diet, route of infusion and the interaction between diet and route of infusion as the fixed effects. Day of isotope infusion was tested as a covariate for the arginine infusion data and was included in the model only when the effect was significant ($P < 0.05$). For all other experimental variables, an analysis of variance with piglet nested in diet as the random term was used. When the model $P < 0.05$, pre-planned pair wise comparisons of least squares means were made using the pdiff option, and the two-tailed p-values were used to assess significance.

The relationship between plasma arginine concentration and each of proline to arginine fractional net conversion and arginine synthesis from proline were studied using the correlation procedure of SAS.

5.3 **Results**

5.3.1 *Piglet performance*

All piglets remained active and healthy throughout the entire trial. For 1 piglet in each of the +Pro, +Orn, +Cit and +Arg groups, the arginine isotope used was degraded. This degradation was confirmed with HPLC and fraction collection analysis of the isotope revealing that only a negligible portion of the total radioactivity in the isotope sample was associated with the arginine peak. Therefore the results from the arginine infusions for these piglets could not be used. A new batch of arginine isotope was used for all subsequent piglets. Thus, for all results involving the ig and iv arginine infusions, results from 4 of 5 piglets for the +Pro, +Orn, +Cit and +Arg treatment groups were used. Based on a priori power calculations, using our previous data (11,12), four values per treatment group is more than adequate to detect significant differences between the diets for arginine flux, first-pass splanchnic arginine extraction, arginine conversion to urea, first-pass splanchnic arginine to urea conversion and $Q_{\text{proline to arginine}}$.

There were no differences among treatment groups with regards to piglet weight at the initiation of test diet infusion (pooled mean = 1.94 kg, pooled SE = 0.09 kg), rate of weight gain during test diet administration [pooled mean = 93 g/(kg·d), pooled SE = 7 g/(kg·d)] and final piglet weight (pooled mean = 2.66 kg, pooled SE = 0.11 kg). Based on our previous results (12) and because of the short duration of the trial, we did not expect to see differences in body weight or weight gains.

5.3.2 *Plasma amino acid concentration*

With the exceptions of alanine, aspartate, glutamate, histidine and lysine, the d 7 plasma concentrations of all amino acids measured were affected by diet ($P < 0.05$) (**Table 5.2**). In general, piglets receiving the +Cit and +Arg diets had a similar plasma amino acid profile. The reasons for the differences in the plasma concentrations of the indispensable amino acids are unclear, and require further investigation, though they may be related to changes in protein synthesis. However, similar differences in plasma methionine and threonine concentrations have been previously described in piglets receiving generous and deficient arginine diets (11), and therefore it does appear that these two amino acids are particularly affected by whole-body arginine status. We have previously found higher plasma asparagine and glutamine concentrations in piglets receiving a deficient versus generous arginine diet (11,12), presumably due to their roles as ammonia scavengers.

Of particular interest are the plasma concentrations of the amino acids that are involved in the proline to arginine synthetic pathway. Plasma proline concentrations in the +Pro piglets were 0.7 to 1.1-fold greater than the proline concentrations in those receiving the other diets ($P < 0.05$) (**Table 5.2**). Ornithine concentrations were similar and higher ($P < 0.05$) in piglets receiving the +Orn, +Cit and +Arg diets than in those receiving the basal and +Pro diets. The +Cit piglets had plasma citrulline concentrations that were ~10-fold greater than the citrulline concentrations in the other treatment groups ($P < 0.05$) (**Table 5.2**). Piglets receiving the basal, +Pro and +Orn diets had significantly ($P < 0.05$) lower plasma arginine concentrations than those receiving the +Cit and +Arg diets (**Table 5.2**).

Table 5.2 Plasma amino acid concentrations at d 7 (μmol/L) in piglets fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

			Diet				
Amino acid	**Basal**	**+Pro**	**+Orn**	**+Cit**	**+Arg**	**Pooled SE**	**p-value**
Indispensable							
Histidine	64	48	69	60	52	9	NS
Isoleucine	114^{ab}	103^{a}	134^{ab}	196^{c}	155^{bc}	17	<0.01
Leucine	254^{bc}	220^{abc}	290^{c}	201^{ab}	149^{a}	29	<0.05
Lysine	301	254	293	250	256	36	NS
Methionine	96^{a}	87^{a}	93^{a}	176^{b}	177^{b}	13	<0.0001
Phenylalanine	65^{a}	64^{a}	79^{ab}	91^{b}	67^{a}	6	<0.05
Threonine	381^{a}	504^{a}	515^{a}	1254^{b}	1208^{b}	101	<0.0001
Tryptophan	26^{a}	23^{a}	17^{a}	25^{a}	39^{b}	4	<0.01
Valine	254^{a}	237^{a}	297^{a}	445^{b}	383^{b}	29	<0.01
Conditionally Indispensable							
Arginine	23^{a}	17^{a}	25^{a}	211^{b}	232^{b}	29	<0.0001
Cystine	10^{ab}	10^{a}	14^{c}	11^{ab}	13^{bc}	1	<0.05
Glutamine	428^{c}	315^{bc}	305^{b}	75^{a}	86^{a}	39	<0.0001
Glycine	2801^{c}	2215^{bc}	1805^{ab}	1590^{ab}	1433^{a}	233	<0.01
Proline	603^{a}	1291^{b}	741^{a}	779^{a}	676^{a}	108	<0.01
Taurine	142^{ab}	119^{a}	132^{ab}	183^{c}	160^{bc}	12	<0.01
Tyrosine	85^{ab}	75^{a}	85^{ab}	123^{c}	108^{bc}	9	<0.01
Dispensable							
Alanine	932	758	726	970	759	113	NS
Asparagine	47^{b}	37^{b}	41^{b}	16^{a}	20^{a}	4	<0.0001
Aspartate	13	10	14	12	10	2	NS
Citrulline	65^{a}	66^{a}	62^{a}	698^{b}	61^{a}	47	<0.0001
Glutamate	143	123	132	135	167	22	NS
Hydroxyproline	107^{ab}	99^{a}	103^{ab}	150^{c}	133^{bc}	11	<0.05
Ornithine	56^{a}	42^{a}	136^{b}	115^{b}	140^{b}	20	<0.01
Serine	569^{d}	425^{ab}	482^{cd}	433^{bc}	318^{ab}	46	<0.05

[1] Values are least square means, n = 5. Means in a row without a common letter differ, $P < 0.05$.

5.3.3 *Plasma ammonia and urea concentrations*

Plasma ammonia concentration was used as a measurement of the ability of the urea cycle to convert ammonia, from amino acid catabolism, to urea for excretion. Higher plasma ammonia concentrations were an indication of impaired urea cycle function. Day ($P = 0.0002$), diet ($P < 0.0001$) and the interaction between diet and day ($P = 0.04$) all had a significant effect on plasma ammonia concentrations (**Table 5.3**). With the initiation of test diet infusion, there was a significant ($P < 0.05$) increase in plasma ammonia concentrations within the first twenty-four hours in piglets receiving the basal, +Pro and +Orn diets, while there was no change ($P > 0.05$) in the plasma ammonia concentration in piglets receiving the +Cit and +Arg diets for the duration of test diet infusion. Therefore, the basal, +Pro and +Orn diets had higher plasma ammonia concentrations than those receiving the +Cit and +Arg diets from the morning of day 4 onwards (**Table 5.3**).

Plasma urea concentration was used as an indirect measure of protein synthesis and whole-body amino acid catabolism. When protein synthesis was higher, due to arginine no longer being limiting to protein synthesis, then fewer of the other dietary amino acids would have been in excess, resulting in a lower amount of amino acid catabolism and a lower need for urea synthesis. Both diet ($P = 0.0005$) and day ($P < 0.0001$) significantly affected plasma urea concentrations (**Table 5.4**). For all piglets in all diet groups, there was an increase in plasma urea concentrations over time ($P < 0.05$); however, by day 7, piglets receiving the basal, +Pro and +Orn diets had higher plasma urea concentrations ($P < 0.05$) than those receiving the +Cit and +Arg diets (**Table 5.4**).

Table 5.3 Plasma ammonia concentrations (μmol/L) in piglets enterally-fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

			Diet			
Day	**Basal**	**+Pro**	**+Orn**	**+Cit**	**+Arg**	**Pooled SE**
3	49.15^{*}	53.60^{*}	51.75^{*}	62.15	66.05	7.36
4	$123.30^{c\dagger}$	$91.45^{b\dagger}$	$93.60^{b\dagger}$	60.15^{a}	58.50^{a}	8.44
5	$115.90^{b\dagger}$	$104.45^{b\dagger}$	$98.50^{b\dagger}$	53.15^{a}	67.30^{a}	10.56
6	$110.60^{b\dagger}$	$101.85^{b\dagger}$	$93.50^{b\dagger}$	60.90^{a}	63.80^{a}	9.70
7	$104.85^{b\dagger}$	$98.20^{b\dagger}$	$105.13^{b\dagger}$	67.40^{a}	70.50^{a}	8.53

[1] Values are least square means, n = 5. Means in a row with subscripts without a common letter differ, $P < 0.05$. Means in a column with subscripts without a common symbol differ, $P < 0.05$.

Table 5.4 Plasma urea nitrogen concentrations (mmol/L) in piglets enterally-fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

			Diet			
Day	**Basal**	**+Pro**	**+Orn**	**+Cit**	**+Arg**	**Pooled SE**
3	0.60^{*}	0.72^{*}	0.82^{*}	0.69	0.78	0.27
4	0.96^{*}	1.06^{*y}	$1.28^{*\dagger}$	1.17	1.09	0.27
5	$1.91^{b\dagger}$	$1.55^{ab\dagger\S}$	$1.93^{ab\dagger\S}$	1.29^{ab}	0.99^{a}	0.27
6	$2.07^{b\dagger}$	$1.62^{ab\dagger\S}$	$2.12^{b\S}$	1.30^{a}	1.02^{a}	0.27
7	$2.26^{b\dagger}$	$2.22^{b\S}$	$2.33^{b\S}$	1.24^{a}	1.22^{a}	0.27

[1] Values are least square means, n = 5. Means in a row with subscripts without a common letter differ, $P < 0.05$. Means in a column with subscripts without a common symbol differ, $P < 0.05$.

5.3.4 *Proline flux and proline conversion to arginine.*

Intragastric proline flux was highest (P < 0.05) in the +Pro piglets, followed by those receiving +Orn, +Arg, +Cit and basal diets (**Table 5.5**). When comparing the intragastric proline fluxes between the +Orn, +Arg, +Cit and basal diets, only the difference between the +Orn and basal piglets was significant (P < 0.05) (**Table 5.5**). Whole-body proline fractional net conversion to arginine was greatest in piglets receiving the +Pro diet, followed by those receiving the basal and +Orn diets, and was lowest in piglets in the +Cit and +Arg groups (P < 0.05) (**Table 5.5**).

5.3.5 *Arginine flux and first-pass splanchnic arginine extraction*

Day of isotope infusion did not have an effect (P > 0.05) on any of the arginine kinetic parameters studied, and thus was not included as a covariate in any of the statistical models used. Intragastric and intravenous arginine fluxes were higher in piglets receiving the +Cit and +Arg diets, than in those receiving the other 3 diets (P < 0.05) (**Table 5.6**). In addition, the intragastric arginine flux was higher (P < 0.05) in the piglets receiving the +Cit diet than in those receiving the +Arg diet (**Table 5.6**). During first-pass splanchnic metabolism, 45-56% of dietary arginine was extracted and the amount of extraction was not affected by dietary treatment (P > 0.05) (**Table 5.6**).

5.3.6 *Arginine conversion to urea.*

Arginine fractional net conversion to urea, calculated using both the intravenous and intragastric infused arginine isotope, was lower in piglets receiving the basal, +Pro and +Orn diets than in those receiving the +Cit and +Arg diets (**Table 5.6**). The

Table 5. 5 Plasma proline and arginine kinetics during the intragastric [U-^{14}C]proline infusion in piglets enterally-fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

	Diet						
	Basal	**+Pro**	**+Orn**	**+Cit**	**+Arg**	**Pooled SE**	**p-value**
Intragastric proline flux [μmol/(kg·h)]	511^{a}	834^{c}	649^{b}	552^{ab}	594^{ab}	38	<0.0001
Intragastric fractional net conversion of proline to arginine (% of arginine flux)	26^{b}	44^{c}	23^{b}	6^{a}	6^{a}	3	<0.0001

[1] Values are least square means, n = 5. Means in a row with subscripts without a common letter differ, P < 0.05.

Table 5.6 Plasma arginine kinetics during the intravenous and intragastric [guanido-^{14}C]arginine infusions in piglets enterally-fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

	Diet					
	Basal *n* = 5	**+Pro** *n* = 4	**+Orn** *n* = 4	**+Cit** *n* = 4	**+Arg** *n* = 4	**p-value**
			intragastric arginine infusion			
Arginine flux [μmol/(kg·h)]	290 ± 27^{a}	255 ± 31^{a}	279 ± 31^{a}	780 ± 31^{c}	661 ± 31^{b}	<0.0001
Fractional net conversion of arginine to urea (% of urea flux)	6.1 ± 3.6^{a}	5.5 ± 4.0^{a}	5.0 ± 4.0^{a}	38.7 ± 4.0^{b}	44.0 ± 4.0^{b}	<0.0001
			Intravenous arginine infusion			
Arginine flux [μmol/(kg·h)]	123 ± 27^{a*}	122 ± 31^{a*}	126 ± 31^{a*}	356 ± 31^{b*}	361 ± 31^{b*}	<0.0001
Fractional net conversion of arginine to urea (% of urea flux)	2.6 ± 3.6^{a}	2.0 ± 4.0^{a}	1.7 ± 4.0^{a}	31.4 ± 4.0^{b*}	31.7 ± 4.0^{b*}	<0.0001
			First-pass splanchnic			
Arginine extraction (%)[2]	56 ± 7	51 ± 8	52 ± 8	54 ± 8	45 ± 8	NS
Fractional net conversion of arginine to urea (% of urea flux)[3]	3.5 ± 3.9	3.4 ± 4.4	3.3 ± 4.4	7.3 ± 4.4	12.3 ± 4.4	NS

[1] Values are least square means ± standard error. Means in a row with subscripts without a common letter differ, $P < 0.05$. *Within a diet, intravenous value is significantly different from the intragastric value, $P < 0.05$. [2] First-pass splanchnic arginine extraction was calculated by [(intragastric arginine flux – intravenous arginine flux) ÷ intragastric arginine flux] * 100%. [3] First-pass splanchnic fractional net conversion of arginine to urea was calculated by subtracting the intravenous value from the intragastric value.

intragastric conversion represents the conversion of dietary arginine to urea, while the intravenous conversion represents the conversion of circulating arginine to urea. Intracellularly-derived arginine that is converted to urea is not determined by either of these calculations. There was no effect ($P > 0.05$) of first-pass splanchnic metabolism on the conversion of arginine to urea (**Table 5.6**).

5.3.7 *Arginine synthesis from proline*

Piglets receiving the +Pro diet had the highest rate of arginine synthesis from proline ($P < 0.05$) (**Table 5.7**), and piglets receiving the +Arg diet had the lowest rate of arginine synthesis from proline ($P < 0.05$) (**Table 5.7**). Piglets receiving the basal, +Orn and +Cit diets had intermediate rates of arginine synthesis from proline (**Table 5.7**). There were significant negative correlations between plasma arginine concentrations and both the fractional net conversion of proline to arginine ($r = -0.68$) ($P = 0.0002$) and arginine synthesis from proline ($r = -0.49$) ($P = 0.02$). Therefore, as arginine status improved, proline use for arginine synthesis was spared.

Table 5.7 Whole-body $Q_{\text{proline to arginine}}$ in piglets enterally-fed either the basal diet or the basal diet supplemented with equimolar amounts of proline, ornithine, citrulline, or arginine[1]

	Diet					
	Basal *n = 5*	**+Pro** *n = 4*	**+Orn** *n = 4*	**+Cit** *n = 4*	**+Arg** *n = 4*	**p-value**
			$Q_{\text{proline to arginine}}$[2]			
μmol/(kg·h)	75 ± 9^{b}	107 ± 10^{c}	63 ± 10^{ab}	48 ± 10^{ab}	40 ± 10^{a}	0.001
g/(kg·d)	0.31 ± 0.04^{b}	0.44 ± 0.04^{c}	0.26 ± 0.04^{ab}	0.20 ± 0.04^{ab}	0.16 ± 0.04^{a}	0.001

[1] Values are least square means ± standard error. Means in a row with subscripts without a common letter differ, $P < 0.05$. [2] $Q_{\text{proline to arginine}}$ was calculated by multiplying the intragastric fractional net conversion of proline to arginine (**Table 5.5**) by the intragastric arginine flux (**Table 5.6**).

5.4 **Discussion**

5.4.1 *Citrulline is an effective arginine precursor*

To our knowledge, this study is the first to use an isotopic approach to examine the effect of supplementation of arginine precursors to a deficient arginine diet administered to neonatal piglets. Piglets receiving the +Cit diet had a similar whole-body arginine statue to those receiving the +Arg diet. Arginine flux is equivalent to the rate of arginine entry into the plasma pool from sources: dietary intake, protein breakdown and de novo arginine synthesis (26). Based on the similar plasma ammonia and urea concentrations and arginine flux in the +Cit and +Arg piglets, these two groups of piglets likely had similar rates of protein synthesis and breakdown as they received isonitrogenous diets; therefore, piglets receiving the +Cit diet must have had a rate of *de novo* arginine synthesis that was high enough to compensate for the 1.60 g/(kg·d) difference in arginine intake. Neither proline nor ornithine addition to the basal diet produced comparable results to piglets receiving the +Arg diet. Therefore, the present results clearly illustrate that citrulline is the most effective arginine precursor, in comparison to proline or ornithine.

Previous studies in puppies (13), kittens (15), adult rats (14) and 7-10 kg growing pigs (16) have all concluded that citrulline is a more effective arginine precursor than ornithine. Suckling piglets given twice daily boluses of citrulline had higher plasma arginine concentrations than control suckling piglets (~260 vs. ~140 μmol/L) (27). Wu and colleagues (27) hypothesized that N-acetyl glutamate (NAG), a cofactor for carbamoylphosphate synthetase I (EC number 6.3.4.16) (1), concentrations are low in suckling piglets, and therefore suckling piglets have a low capacity for citrulline synthesis. Arginine is an allosteric activator of NAG synthase (EC number 2.3.1.1) (28), and therefore as arginine concentrations fall, the ability of piglets to synthesize arginine may also decrease due to a lower NAG synthase activity. The basal, +Pro and +Orn piglets had plasma arginine concentrations below the sow-fed reference range (50 – 267 μmol/L) (29); therefore, these piglets may have had an even greater limitation for citrulline and subsequently arginine formation than suckling piglets.

The significantly higher plasma citrulline concentrations in the +Cit versus +Arg piglets, in spite of similar plasma arginine concentrations and arginine flux, suggests that arginine synthesis from citrulline was occurring at a maximal rate, resulting in the accumulation of citrulline in the plasma. Plasma citrulline concentrations were well outside the sow-fed reference range (73 – 151 μmol/L) (29) in the +Cit piglets. Therefore, citrulline levels were likely much higher than the K_m for argininosuccinate synthetase (EC number 6.3.4.5), which is ~60 μmol/L for the human isoform (30). Unlike ornithine and proline, citrulline cannot be metabolized by any other metabolic pathways without first being converted to arginine (28) and this may explain why citrulline was a more effective arginine precursor than either proline or ornithine.

5.4.2 *Ornithine is an ineffective arginine precursor*

Ornithine can be metabolized by three different enzymes: ornithine transcarbamoylase (EC number 2.1.3.3), which catalyzes citrulline formation for use in the urea cycle or for arginine synthesis (28); ornithine amino transferase (OAT, EC number 2.6.1.13) which converts ornithine to P5C which can subsequently be metabolized to α-ketoglutarate or proline (31); and ornithine decarboxylase (EC number

4.1.1.17) for polyamine synthesis. Of these three enzymes, *in vitro* work using week-old piglet enterocytes has shown that the activity of OAT is the highest, (32,33), suggesting that the conversion of ornithine to P5C is the most favored route of ornithine metabolism. Indeed, an *in vivo* study in neonatal piglets receiving no dietary ornithine, found that 25% and 36% of ornithine flux, for intragastrically and intraportally infused ornithine respectively, was oxidized to CO_2 (23). Alternatively, it is also possible that due to the limitation in citrulline formation, as previously discussed, the supplemented ornithine was oxidized to prevent accumulation. There was no difference in plasma proline concentrations between the basal and +Orn piglets (**Table 5.2**), indicating that there was probably not a large conversion of the supplemented ornithine to proline. These results indicate that a large portion of ornithine may have been oxidized, preventing it from being used to support urea cycle function or as an arginine precursor.

The ineffectiveness of ornithine as an arginine precursor could also be due to a limitation in the activity of ORNT1, the mitochondrial membrane transporter. In neonatal mice, ORNT1 activity in both the small intestine and liver increases during the early-postnatal period (34). To the authors' knowledge, there have been no reports of the development of ORNT1 in neonatal piglets; however, if ORNT1 activity is low in the age of piglets in the present study, then there may have been a limitation in the ability of the supplemental ornithine to be transported to mitochondria for citrulline formation.

5.4.3 *Proline is an ineffective arginine precursor*

Although proline is the major dietary precursor for arginine in week-old piglets (9,11), the results from the +Pro and basal piglets show that it is not the optimal arginine precursor. We have not previously determined the amount of proline required by piglets to support the maximal rate of arginine synthesis, although the amount is likely equal or less than the 1.25 g/(kg·d) proline provided in the basal diet. Dillon et al. (35) found that there is a substantial conversion of proline to P5C, but only a very small portion, 5-6%, of proline-derived P5C is converted to arginine in 2-week old piglet enterocytes *in vitro*. Compared to other ages of piglets, week-old piglet enterocytes have the lowest amount of total proline to ornithine conversion (36). These *in vitro* findings strongly support our *in vivo* finding that there is a limit to proline use for arginine synthesis in week-old piglets.

5.3.4 *Effective arginine precursors spare the use of proline for arginine synthesis*

We have previously observed that piglets fed a generous arginine diet have a lower conversion of proline to arginine than piglets fed inadequate arginine diets (11,12); therefore, when arginine status is favorable, proline use for arginine synthesis is spared. Arginine and citrulline were both effective arginine precursors and therefore had the lowest rates of conversion of proline to arginine. Piglets fed the +Orn diet had a greater reliance on proline for arginine synthesis than the +Cit and +Arg piglets, because ornithine was not an effective arginine precursor. Proline to arginine conversion was highest in piglets receiving the +Pro diet, even higher than the level observed for the basal diet, which may indicate that the basal diet did not contain enough proline to maximally support endogenous arginine synthesis. However, none of the other parameters studied with regards to whole-body arginine status (**Table 5.2-5.4, Table 5.6**) were improved by proline supplementation. The reasons for this contradiction in the effectiveness of supplemental proline as an arginine precursor are unknown. However, the rate of arginine synthesis from proline may have been overestimated in the +Pro piglets because the proline pool was likely substantially larger in the +Pro piglets compared to the other groups, as illustrated by higher plasma proline concentrations and proline flux. In summary, citrulline and arginine, but not proline or ornithine addition to an arginine deficient diet spares the use of proline for arginine synthesis.

5.4.5 *Splanchnic extraction of dietary arginine.*

We believe that our finding that 52% (Table 5) of dietary arginine is extracted during first-pass splanchnic metabolism is the first to show that arginine intake and status do not affect splanchnic arginine use. The reason why the splanchnic region, which includes the intestine and liver, is extracting a substantial portion of dietary arginine, especially in the cases where piglets were in a deficient arginine state, is unknown. Piglets in the +Cit and +Arg groups had significant conversion of arginine to urea during first-pass splanchnic metabolism, which was not seen in piglets in the other treatment groups, and this may be a mechanism whereby piglets with a favorable arginine status can maintain their plasma arginine concentrations within a certain range. However, the formation of urea from arginine resulted in the removal of the radioactive label from arginine. Although the resulting ornithine may have been recycled back to arginine, this arginine was calculated as extracted because it no longer carried the label. Therefore, the splanchnic extraction of arginine in piglets receiving the +Cit and +Arg diets may have been over-estimated due to the production of urea. Other possible metabolic fates of the extracted arginine include the use for splanchnic nitric oxide, polyamine and/or protein synthesis. Because of the importance of arginine in supporting piglet growth (2) and implications in preventing certain neonatal pathologies (37), a thorough understanding of the uses of dietary arginine during first-pass splanchnic metabolism is necessary.

5.4.6 *Contribution of dietary arginine to whole-body arginine flux*

Using the 52% calculated value for first-pass splanchnic extraction to calculate the amount of dietary arginine entering general circulation, in combination with the intravenous arginine fluxes (**Table 5.6**), we calculated the portion of flux derived from dietary arginine for each group. Dietary intake represented 19, 19, 18, 6 and 57% of arginine flux in piglets receiving the basal, +Pro, +Orn, +Cit and +Arg diets, respectively. This finding illustrates the importance of endogenous sources of arginine, which may include arginine from both protein breakdown or de novo arginine synthesis, for arginine flux, particularly when dietary arginine is limiting. Regardless of whole-body arginine status, the splanchnic region extracted a minimum of 25 μmol/(kg·h) and this value was much higher when there was a generous intake of arginine.

5.4.7 *Conclusion*

In conclusion, citrulline was the most effective arginine precursor, while there was a limit to the extent that either proline or ornithine could be used as an arginine precursor. The formation or availability of citrulline at the site of endogenous arginine synthesis may limit arginine synthesis in piglets receiving an arginine deficient diet. The percent of dietary arginine extracted during first-pass splanchnic extraction is independent of whole-body arginine status and accounts for approximately 52% of intake. Arginine is critical for the growth and health of neonates; therefore, a complete knowledge of the mechanisms that control its synthesis are necessary in order to optimize diets for young pigs, human neonates and other young mammals.

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6.0 CO-ADMINISTRATION OF ORNITHINE AND α-KETOGLUTARATE IS NO MORE EFFECTIVE THAN ORNITHINE ALONE AS AN ARGININE PRECURSOR IN PIGLETS ENTERALLY-FED AN ARGININE-DEFICIENT DIET[1]

6.1 Introduction

When equimolar amounts of the arginine precursors proline, ornithine and citrulline were individually added to an arginine deficient diet given to enterally-fed neonatal piglets, only citrulline was as effective as arginine in improving whole-body arginine status (1). These data suggested that there was a limitation in the ability of enterally-administered ornithine to be used as an arginine precursor, and that the supplemental ornithine was being converted to metabolites other than arginine (1). Other than arginine, ornithine may be converted to numerous other metabolites including: polyamines and pyrroline-5-carboxylate (P5C) (2). P5C can subsequently be converted to either proline or to glutamate, which can then be converted to either glutamine or α-ketoglutarate, which enters the tricarboxylic acid cycle (2).

Ornithine α-ketoglutarate (OKG) is an ionic salt that dissociates in solution into 2 moles of ornithine and 1 mole of α-ketoglutarate, and has been used clinically in many situations to improve nitrogen retention (for review, see 3). A study in rats following fracture trauma (4) found that OKG had greater positive effects on nitrogen retention than either of the OKG components, α-ketoglutarate or ornithine, separately. Moreover, in healthy adults receiving a bolus dose of either OKG, ornithine-HCl or calcium α-ketoglutarate, only OKG administration resulted in a significant increase in plasma arginine and proline concentrations, whereas the increase in plasma glutamate concentrations was pronounced only when ornithine or α-ketoglutarate alone was administered (5).

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[1] A version of this chapter has been published. Urschel et al. 2007. *J Nutr*. 137: 55-62. A portion of this chapter has been accepted for publication in *Livestock Science*.

The proposed mechanism for the biochemical effects of OKG in humans is that, by simultaneously administering two components that are metabolically-related by a series of reversible reactions, the equilibrium of ornithine metabolism is shifted away from glutamate and α-ketoglutarate production and towards the production of the other ornithine metabolites including proline, arginine and polyamines (3,5). To our knowledge, neither OKG metabolism nor this proposed mechanism have been tested *in vivo* in neonates using isotopically-labeled compounds. If the proposed mechanism of OKG metabolism occurs in neonatal piglets, then ornithine co-administered with α-ketoglutarate (OKG) should improve whole-body arginine status in enterally-fed piglets receiving an arginine deficient diet.

The first objective of the present study was to examine ornithine metabolism in piglets receiving an arginine deficient basal diet or the basal diet supplemented with either α- ketoglutarate (+α-KG), ornithine (+Orn), or OKG as α-ketoglutarate and ornithine (+α -KG/+Orn) in a 1:2 molar proportion. Specifically, we wanted to determine whether the OKG improved the use of ornithine for arginine synthesis relative to ornithine administration alone.

Ornithine aminotranferase (OAT; EC number 2.6.1.13) is the enzyme responsible for the conversion between P5C and ornithine, and is primarily localized to the small intestine of piglets (6). In 7-day old suckling piglets, the activity of OAT is approximately 1-fold greater in the direction of P5C than in the direction of ornithine (7,8); this may further explain why ornithine does not seem to be used effectively as an arginine precursor (1). First-pass intestinal metabolism is an important site for both ornithine (9) and arginine (10) synthesis in enterally-fed neonatal piglets. Furthermore, only about 10% of enterally-administered α-ketoglutarate is absorbed into the portal vein, suggesting extensive first-pass intestinal α-ketoglutarate metabolism (11). First-pass intestinal metabolism was hypothesized to important, not only for ornithine metabolism, but also for the metabolic interaction between α-ketoglutarate and ornithine. The second objective of the present study was to elucidate the role of first-pass intestinal metabolism in ornithine metabolism in neonatal piglets by infusing ornithine radioisotopes both intragastrically and intraportally.

6.2 Materials and methods

6.2.1 *Animals and surgical procedures*

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee for the University of Alberta. Twenty intact male Landrace/ Large White/ Duroc piglets (Hypor, Regina SK, Canada) (1.5 – 2.0 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant a gastric catheter for diet and intragastric isotope infusion, an umbilical vein catheter advanced to the portal vein for intraportal isotope infusion, and a femoral vein catheter for blood sampling.

Twenty minutes prior to surgery (d 0), piglets were given an intramuscular injection of atropine sulfate (0.05 mg/kg; MTC Pharmaceuticals, Cambridge ON, Canada), an intramuscular injection of ampicillin sodium (20 mg/kg; Novopharm, Toronto ON, Canada), and a subcutaneous injection of meloxicam (0.2 mg/kg; Metacam 0.5% injection, Boehringer Ingelheim, Burlington ON, Canada). Piglets were then masked, and pre-dosed with oxygen, followed by initial anaesthesia with halothane (halothane, B.P.; MTC pharmaceuticals, Cambridge ON, Canada). Once lightly anaesthetized, piglets were intubated, and anaesthesia was maintained throughout surgery using 1.5-2.5% isoflurane (AErrane; Baxter, Mississauga ON). This pre-surgical procedure is a modification to previously described methods (12) and has resulted in more stable piglet vital signs during surgery. Surgical procedures, post-surgical care and piglet housing were recently described (12).

6.2.2 *Diets and treatment groups*

A complete elemental diet, designed to meet the nutrient requirements of neonatal piglets (12), was continuously infused enterally via the gastric catheter using pressure-sensitive infusion pumps (IVAC 560; IVAC, San Diego CA, USA). Diet composition, preparation, and piglet adaptation to the enteral diet were all as previously described (1,12,13).

On the morning of d 3 piglets were randomly assigned to one of four test diets (n = 5/diet): an arginine-deficient basal diet (basal; 0.73 g/L arginine in the base diet), or the basal diet supplemented with either α-ketoglutarate (+α-KG; 2.47 g/L α-ketoglutaric acid), ornithine (+Orn; 5.70 g/L ornithine-HCl), or both α-ketoglutarate and ornithine (α-KG/+Orn; 2.47 g/L α-ketoglutaric acid and 5.70 g/L ornithine-HCl). Details of the basal diet composition were recently described (1). The α-KG/+Orn diet had a molar ratio of ornithine to α-ketoglutarate of 2:1; this is equivalent to OKG because OKG dissociates in solution into its two components. To ensure that the diets were isonitrogenous, the concentrations of alanine (9.64 g/L in the basal and +α-KG diets; 6.63 g/L in the +Orn and +α-KG/+Orn diets) and glycine (3.61 g/L in the basal and +α-KG diets; 1.07 g/L in the +Orn and +α-KG/+Orn diets) were adjusted. The +α-KG, +Orn and + α-KG/+Orn base solutions were used within 1-2 weeks of being mixed to ensure the stability of the ornithine and α-ketoglutarate in solution. Previous work has shown that both ornithine and α-ketoglutarate are stable in solution for at least 21 days when stored at 4 °C (14).

6.2.3 *Blood sampling*

Blood samples (2 mL) were collected every 24 hours, beginning immediately prior to the allocation to test diets on d 3 and continuing until the end of the trial. The daily blood samples were used for the determination of plasma ammonia and urea nitrogen concentrations, and the blood sample taken on d 7 was also used for the determination of plasma amino acid concentrations. As described under *constant tracer infusions*, additional blood samples were taken during tracer infusions on d 5, d 6 and d 7.

6.2.4 *Constant tracer infusions*

On the morning of d 5, ornithine kinetics were determined by a primed [481 kBq (13 μCi)/kg], constant [370 kBq (10 μCi)/(kg·h)] infusion of L-[1-^{14}C]ornithine (1.96 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). Half of the piglets in each dietary treatment were given an intragastric infusion of the isotope via the gastric catheter, and the other piglets received an intraportal infusion via the umbilical catheter. On d 7, piglets were infused with L-[1-^{14}C]ornithine via the route of infusion that they did not receive on d 5. To enable $^{14}CO_2$ collection, for the measurement of [1-^{14}C]ornithine oxidation, piglets were contained in plexi-glass boxes (30 cm x 60 cm x 45 cm high) for the duration of both of these isotope infusions. The isotope was infused over a 6.5 h period, and blood (1 mL) was sampled at 0, 60, 120, 180, 240, 270, 300, 330, 360 and 390 min. On d 7, additional samples were taken one hour (–60 min) and 30 min (–30 min) prior to the start of isotope infusion to correct for the background specific activity (SA) of arginine in the blood. Breath samples were collected every 30 minutes for the duration of the isotope infusion for the determination $^{14}CO_2$, using a previously described procedure (15). On d 7, an additional hour of breath sampling was completed prior to the initiation of isotope infusion in order to correct for background $^{14}CO_2$ production. Infusing the ornithine isotope both intragastrically and intraportally enabled us to examine the effects of first-pass intestinal metabolism on ornithine metabolism (9,10).

On the morning of d 6, arginine kinetics were determined by a primed [111 kBq (3 μCi)/kg], constant [185 kBq (5 μCi)/(kg·h)] infusion of L-[guanido-^{14}C]arginine (2.11 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). Glutamate kinetics were simultaneously determined using a primed [740 kBq (20 μCi)/kg], constant [370 kBq (10 μCi)/(kg·h)] infusion of L-[3,4-^{3}H]glutamate (1480 GBq/mmol; Moravek Biochemicals, Inc., Brea CA, USA). Both isotopes were infused intragastrically for 6.5 hours. Blood (1 mL) was sampled at –60, -30, 0, 60, 120, 180, 240, 270, 300, 330, 360 and 390 min. The infusion doses and periods for all infusions were based on previous experiments (1,9,10,12). Diets were infused continuously throughout all isotope infusions.

At the end of the d 7 infusion, piglets were euthanized with an injection of 500 mg of pentobarbital sodium (Euthansol, 340 g/L; Schering Canada Inc., Pointe Claire PQ) into the femoral vein catheter.

6.2.5 *Analytical procedures*

The radioactivity of the breath samples collected during the [1-^{14}C]ornithine infusions was determined by combining a 1 mL aliquot of the collected breath sample with 6 mL of scintillant (Atomlight; PerkinElmer Life and Analytical Sciences, Boston

MA, USA), and counting the samples on a scintillation counter (Tri-Carb 4000 series, Canberra Packard, Canada).

Plasma amino acid concentrations and the SA of arginine, glutamate, ornithine ([1-^{14}C]ornithine infusion samples only) and proline ([1-^{14}C]ornithine infusion samples only) were measured by reverse-phase HPLC using phenylisothiocyanate derivatives and post-column collection of the radioactive derivatives of arginine, glutamate, leucine, ornithine and proline, as previously described (1,16,17).

Plasma ammonia (Reference 200-02; Diagnostic Chemical Limited, Charlottetown PEI, Canada) and urea nitrogen (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA) concentrations were determined every 24 h during test diet infusion (d 3 – d 7) using spectrophotometric assays.

6.2.6 *Calculations*

The fractional net conversions of the precursor (ornithine) to product metabolite (either arginine, glutamate or proline), whole-body fluxes for the intragastrically infused ornithine, glutamate and arginine and the intraportally infused ornithine, and the the absolute conversion of precursor (ornithine) to product (arginine, glutamate or proline) ($Q_{\text{ornithine to product aa}}$) were all calculated using recently described formulas (1).

The calculated flux values included the amino acids entering the plasma pool through the diet, *de novo* synthesis and protein breakdown, or leaving the pool through protein synthesis, oxidation or conversion to other metabolites. The intragastric fluxes are influenced by both first-pass splanchnic metabolism and the metabolism by other peripheral tissues (such as muscle, kidney, lung, and intestinal metabolism of arterial substrates), whereas intraportal fluxes exclude the influences of first-pass intestinal metabolism. Although the intragastric proline flux was not measured in the present study, it was previously determined in a similar study, using similar piglets receiving the identical basal and +Orn diets and identical methods (1). Therefore, the previously determined proline flux value of 511 μmol/(kg·h) was used for piglets receiving the basal and +α-KG diets, and a proline flux value of 649 μmol/(kg·h) was used for piglets receiving the +Orn and + α-KG/+Orn diets.

First-pass intestinal conversion of ornithine to product amino acid (arginine, glutamate, proline) was calculated within piglet by subtracting the intraportal from the intragastric value. The first-pass intestinal extraction of ornithine was calculated within piglet using the approach of Castillo et al. (18) which is as follows:

First-pass intestinal extraction (%) = 100 – (ornithine $\text{flux}_{\text{intraportal}}$ [μmol/(kg·h)] / ornithine $\text{flux}_{\text{intragastric}}$ [(μmol/(kg·hr)]).

Ornithine oxidation was calculated using the series of formulas as previously described (9,15).

6.2.7 *Statistical analyses*

Unless otherwise stated, all data were analyzed using the mixed procedure of SAS Version 8.3 (SAS Institute, Cary NC, USA), and differences were considered statistically significant if $P < 0.05$.

The dependent variables plasma ammonia and plasma urea nitrogen were analyzed using repeated measures analysis where the fixed effect was diet and the random variables were piglet nested in diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis based on Schwarz's Bayesian Criterion. When the effects were significant ($P < 0.05$), pre-planned comparisons, between diets but within a day or within a diet but between days, of least squares means were made using the pdiff test, which is the only two-tailed pairwise comparison test used by the mixed procedure.

The results from the intragastric and intraportal ornithine infusions were analyzed using a 4 x 2 factorial design with the diet, route of infusion and the interaction between diet and route of infusion as the fixed effects. Day of isotope infusion was tested as a covariate for the ornithine infusion data and was included in the model only when the effect was significant ($P < 0.05$). For all other experimental variables, an analysis of variance with piglet nested in diet as the random term was used. When the model was significant ($P < 0.05$), pre-planned pair wise comparisons, between diets but within a route of infusion or within a diet but between routes of infusion, of least squares means were made using the pdiff option, and the two-tailed p-values were used to assess the significance ($P < 0.05$) of differences between the dietary treatment groups.

6.3 **Results**

6.3.1 *Piglet performance*

One piglet receiving the +α-KG/+Orn treatment was removed from the trial on the evening of d 6 due to severe gastric and intestinal distention, likely due to gut stasis; post-mortem inspection of the piglet did not reveal the cause of the gut stasis. Therefore, n = 4 for the +α-KG/+Orn diet for all d 7 measurements, which included the intraportal ornithine infusion. Based on a priori power calculations, using our previous data (1,10,12), four values in this treatment group was more than adequate to detect significant differences in plasma amino acid, ammonia and urea concentrations, ip ornithine metabolism, and first-pass intestinal ornithine metabolism.

On d 3, there were no differences in piglet weight at the initiation of test diet infusion (pooled mean = 2.16 kg, pooled SE = 0.13 kg). Dietary treatment did not affect ($P > 0.05$) on the rate of weight gain during test diet administration [pooled mean = 85 g/(kg·d), pooled SE = 12 g/(kg·d)] or final piglet weight (pooled mean = 2.90 kg, pooled SE = 0.14 kg). Due to the short duration of the trial, and based on our previous results (1,12), we did not expect to see differences in body weight or weight gains.

6.3.2 *Plasma amino acid, ammonia and urea concentrations*

The only plasma amino acid concentrations that were affected by diet were ornithine and glutamate ($P < 0.05$). Piglets receiving the two diets containing ornithine had plasma ornithine concentrations that were 2.7 – 4.8 fold higher (+Orn: 213 μmol/L; +α-KG/+Orn: 168 μmol/L) than in piglets not receiving dietary ornithine (basal: 46 μmol/L; +α-KG: 37 μmol/L; pooled SE: 17 μmol/L). Plasma glutamate concentrations were greatest in piglets receiving the +Orn diet (102 µmol/L) and lowest in the +α-KG piglets (52 µmol/L), with intermediate values in the piglets receiving the basal (73 µmol/L) and +Orn/+ α-KG (58 µmol/L; pooled SE: 8 µmol/L) diets. The diets tended ($P = 0.08$) to affect on the plasma proline concentration, with +Orn piglets having the highest plasma proline concentration (886 µmol/L), followed by the +α-KG/+Orn (785 µmol/L), basal (624 µmol/L), and +α-KG (478 µmol/L; pooled SE: 112 µmol/L) piglets.

The use of plasma ammonia and urea concentrations as indicators of whole-body metabolism has been recently discussed (1). There was a significant effect of day ($P = 0.0002$), but not for diet ($P = 0.23$) or their interaction ($P = 0.42$), on plasma ammonia concentrations (**Table 6.1**). Piglets receiving the +Orn and +a-KG/+Orn diets, experienced only transient increases in plasma ammonia concentrations relative to the d 3 levels, while piglets in the other two groups had elevated plasma ammonia concentrations from d 4 onwards.

Diet ($P < 0.0001$) and day ($P < 0.0001$), but not their interaction ($P = 0.78$) affected plasma urea concentrations (**Table 6.1**). The plasma urea concentrations were higher from d 4 onwards than on d 3 ($P < 0.05$) in piglets in the basal, +α-KG and + α-KG/+Orn groups. In addition, piglets receiving the +Orn diet had lower plasma urea concentrations than piglets receiving the +α-KG diet from d 4 onwards ($P < 0.05$).

6.3.3 *Arginine and glutamate fluxes*

The intragastric arginine flux, which includes the effects of both first-pass splanchnic and peripheral metabolism, was not affected by diet [pooled mean: 369 µmol/(kg·h); pooled SE: 71 µmol/(kg·h)]. Diet tended ($P = 0.08$) to affect the

Table 6.1 Plasma ammonia and urea concentrations in piglets enterally-fed either the basal diet or the basal diet supplemented with α-ketoglutarate, ornithine, or both[1]

Day	Diet			
	Basal	**+α-KG**	**+Orn**	**+ α -KG/ +Orn**
Plasma ammonia				
		μmol/L		
3	$41 \pm 13^{*}$	$72 \pm 13^{*}$	$48 \pm 13^{*}$	$64 \pm 13^{*\dagger}$
4	$111 \pm 22^{\dagger}$	$146 \pm 22^{\dagger}$	$92 \pm 22^{*\dagger}$	$54 \pm 22^{*}$
5	$97 \pm 19^{\dagger}$	$110 \pm 19^{\dagger}$	$96 \pm 19^{\dagger}$	$96 \pm 19^{*\dagger}$
6	$145 \pm 26^{\dagger}$	$135 \pm 26^{\dagger}$	$87 \pm 26^{*\dagger}$	$138 \pm 26^{\dagger}$
7	$118 \pm 13^{\dagger}$	$119 \pm 13^{\dagger}$	$81 \pm 13^{*\dagger}$	$95 \pm 15^{*\dagger}$
Plasma urea				
		mmol/L		
3	$0.82 \pm 0.49^{*}$	$1.11 \pm 0.49^{*}$	0.76 ± 0.49	$0.98 \pm 0.49^{*}$
4	$1.59 \pm 0.49^{ab\dagger}$	$2.88 \pm 0.49^{b\dagger}$	1.27 ± 0.49^{a}	$1.94 \pm 0.49^{ab\dagger}$
5	$2.24 \pm 0.49^{ab\dagger\ddagger}$	$3.66 \pm 0.49^{b\ddagger}$	1.42 ± 0.49^{a}	$2.18 \pm 0.49^{ab\dagger}$
6	$2.66 \pm 0.49^{ab\dagger\ddagger}$	$3.64 \pm 0.49^{b\dagger\ddagger}$	1.60 ± 0.49^{a}	$2.52 \pm 0.49^{ab\dagger}$
7	$3.04 \pm 0.49^{ab\ddagger}$	$3.46 \pm 0.49^{b\dagger\ddagger}$	1.51 ± 0.49^{a}	$1.82 \pm 0.5^{ab*\dagger}$

[1] Values are least square means ± SEM, n = 5, except for +α-KG/+Orn on d 7 (n = 4). Means in a row without a common superscript letter differ, $P < 0.05$. Means in a column without a common superscript symbol differ, $P < 0.05$.

intragastric glutamate flux [basal: 5557 µmol/(kg·h); +α-KG: 3706 µmol/(kg·h); +Orn: 5771 µmol/(kg·h); +α-KG/+Orn: 8181 µmol/(kg·h); pooled SE: 1117 µmol/(kg·h)], and piglets receiving the +α-KG diet, the diet which also resulted in the lowest plasma glutamate concentration, had a ~50% lower glutamate flux than piglets in the +α-KG/+Orn group.

6.3.4 *Ornithine flux and first-pass intestinal ornithine extraction*

In all groups, the intragastric flux was greater than the intraportal flux ($P < 0.05$), but the difference between the intragastric and intraportal fluxes was greater in the piglets not receiving any dietary ornithine than in those piglets in the +Orn and +α-KG/+Orn groups ($P < 0.05$) (**Table 6.2**). For both intragastric and intraportal routes of infusion, ornithine flux was greater in the +Orn and +α-KG/+Orn piglets than in the other two treatment groups ($P < 0.05$) (**Table 6.2**). A greater percentage of ornithine was extracted during first-pass intestinal metabolism in basal and + α-KG piglets than in the +Orn and + α-KG/+Orn piglets (**Table 6.2**) ($P < 0.0001$).

6.3.5 *Ornithine oxidation*

The intragastric ornithine oxidation, as a percent of ornithine dose oxidized, was different in all four groups ($P < 0.05$): ornithine oxidation was lowest in piglets receiving the +α-KG diet, followed by the basal and the +Orn groups, and was greatest in piglets receiving the +Orn/+α-KG diet (**Table 6.2**). The intraportal ornithine oxidation was higher in piglets receiving dietary ornithine than in the other two groups of piglets (P <

Table 6. 2 Ornithine flux, oxidation and first-pass intestinal extraction during the [1-^{14}C]ornithine infusions in piglets enterally-fed either the basal diet or the basal diet supplemented with α-ketoglutarate, ornithine, or both α-ketoglutarate and ornithine[1]

	Diet				**P-values**		
	Basal	**+α-KG**	**+Orn**	**+α -KG/ +Orn**	**Diet**	**Route of infusion**	**Diet* Route**
Ornithine flux							
		μmol/(kg h)					
Intragastric	234 ± 27^{a}	241 ± 27^{a}	473 ± 27^{b}	527 ± 27^{b}	<0.0001	<0.0001	NS[2]
Intraportal	$126 \pm 27^{a*}$	$105 \pm 27^{a*}$	$400 \pm 27^{b*}$	$409 \pm 30^{b*}$			
Ornithine oxidized							
		% of dose					
Intragastric	19.6 ± 1.2^{b}	15.4 ± 1.2^{a}	29.8 ± 1.2^{c}	33.5 ± 1.2^{d}	<0.0001	<0.0001	<0.001
Intraportal	$35.5 \pm 1.2^{a*}$	$34.0 \pm 1.2^{a*}$	$39.3 \pm 1.2^{b*}$	$42.5 \pm 1.3^{b*}$			
First-pass intestinal ornithine extraction[3]							
			%				
	45 ± 5^{b}	56 ± 5^{b}	16 ± 5^{a}	16 ± 5^{a}	<0.0001	---	---

[1] Values are least square means ± SEM, n = 5, except for +α-KG/+Orn for the intraportal ornithine infusion (n = 4). Means in a row without a common superscript letter differ, $P < 0.05$. *intraportal ornithine flux or oxidation was significantly different from the intragastric infusion value. [2] Not significant ($P > 0.05$). [3] First-pass intestinal ornithine extraction was calculated by 100 – (ornithine $\text{flux}_{\text{intraportal}}$ / ornithine $\text{flux}_{\text{intragastric}}$).

0.05) (**Table 6.2**). The intraportal oxidation of ornithine was greater than the intragastric rate in all four treatment groups ($P < 0.05$) (**Table 6.2**).

6.3.6 *Ornithine conversion to other metabolites.*

With the exception of the ornithine to glutamate conversion in piglets receiving the +Orn and +α-KG/+Orn diets, the intragastric fractional net conversions of ornithine to arginine, glutamate and proline were greater than the intraportal conversions ($P < 0.05$) (**Table 6.3**). Both the intraportal and intragastric conversions of ornithine to proline were greater in the +Orn and +α-KG/+Orn piglets than in the piglets receiving the other diets ($P < 0.05$) (**Table 6.3**). The fractional net conversion of ornithine to glutamate was not affected by diet, regardless of route of infusion (**Table 6.3**), although the interaction between diet and route of infusion did tend to affect this conversion ($P = 0.06$). Diet did not affect the intragastric fractional net conversion of ornithine to arginine; however, for the intraportal infusion, this conversion was lowest in the +α-KG group, intermediate in the basal group and was greatest in the +Orn and +α-KG/+Orn groups ($P < 0.05$) (**Table 6.3**).

Similar to the fractional net conversion values, the intraportal rates of synthesis of ornithine metabolites were lower than the intragastric synthetic rates ($P < 0.05$), with the exception of the glutamate synthetic rate in piglets receiving +Orn and +α-KG/+Orn diets, where there was no effect of route of infusion (**Table 6.3**). Day was only significant as a covariate ($P = 0.03$) for the arginine synthetic rate, so this was the only statistical model where day was included. Numerous changes in the activities of the enzymes involved in arginine synthesis occur between 7 to 14 days of age in piglet

Table 6.3 Ornithine conversion to arginine, glutamate and proline in piglets enterally-fed either the basal diet or the basal diet supplemented with α-ketoglutarate, ornithine, or both[1]

	Diet				P-values		
	Basal	+α-KG	+Orn	+α -KG/ +Orn	Diet	Route of infusion	Diet* Route
Ornithine conversion to arginine							
Fractional net conversion							
		% of arginine flux from ornithine					
Intragastric	33.1 ± 5.0	47.2 ± 5.0	35.0 ± 5.0	39.7 ± 5.0			
Intraportal	$8.9 \pm 5.0^{*}$	$6.1 \pm 5.0^{*}$	$21.0 \pm 5.0^{*}$	$18.1 \pm 5.7^{*}$	NS	<0.0001	NS
Arginine synthesis from ornithine							
		μmol/(kg h)					
Intragastric[2,3]	147 ± 22	153 ± 22	134 ± 22	125 ± 22	NS	<0.0001	<0.05
Intraportal [2,3]	$36 \pm 22^{*}$	$18 \pm 22^{*}$	$80 \pm 22^{*}$	$65 \pm 24^{*}$			
First-pass intestinal arginine synthesis[4]							
		μmol/(kg h)					
	106 ± 21^{ab}	131 ± 21^{b}	59 ± 21^{a}	48 ± 23^{a}	<0.05	—	—
Ornithine conversion to glutamate							
Fractional net conversion							
		% of glutamate flux from ornithine					
Intragastric	6.09 ± 1.37	6.49 ± 1.37	7.46 ± 1.37	8.14 ± 1.37	<0.05	<0.05	NS
Intraportal	$2.19 \pm 1.37^{ab*}$	$1.72 \pm 1.37^{a*}$	6.13 ± 1.37^{b}	9.38 ± 1.54^{b}			

Glutamate synthesis from ornithine							
		µmol/(kg h)					
Intragastric[2]	273 ± 61^{ab}	213 ± 61^{a}	392 ± 61^{bc}	620 ± 61^{c}	<0.0001	<0.001	NS
Intraportal[2]	$121 \pm 61^{a*}$	$65 \pm 61^{a*}$	298 ± 61^{b}	720 ± 67^{c}			
First-pass intestinal glutamate synthesis[4]							
		µmol/(kg h)					
	152 ± 67	148 ± 67	93 ± 67^{5}	-105 ± 75^{5}	NS	---	---
Ornithine conversion to proline							
Fractional net conversion							
		% of proline flux from ornithine					
Intragastric	9.9 ± 0.7^{a}	10.3 ± 0.7^{a}	23.0 ± 0.7^{b}	22.8 ± 0.7^{b}	<0.0001	<0.0001	<0.05
Intraportal	$3.1 \pm 0.7^{a*}$	$2.9 \pm 0.7^{a*}$	$12.3 \pm 0.7^{b*}$	$12.0 \pm 0.8^{b*}$			
Proline synthesis from ornithine							
		µmol/(kg h)					
Intragastric[2]	51 ± 5^{a}	53 ± 5^{a}	149 ± 5^{b}	148 ± 5^{b}	<0.0001	<0.0001	<0.01
Intraportal[2]	$16 \pm 5^{a*}$	$15 \pm 5^{a*}$	$80 \pm 5^{b*}$	$78 \pm 5^{b*}$			
First-pass intestinal proline synthesis[4]							
		µmol/(kg h)					
	35 ± 6^{a}	38 ± 6^{a}	69 ± 6^{b}	69 ± 6^{b}	<0.001	---	---

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[1] Values are least square means ± SEM, n = 5, except for +α-KG/+Orn for the intraportal rates of synthesis (n = 4). Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from the intragastric rate, $P < 0.05$. [2] Rates of synthesis were calculated, within piglet, by multiplying intragastric or intraportal fractional net conversion value by intragastric flux value for the product amino acid (**Table 6.2** and (1)). [3] Day was significant as a covariate for the arginine synthetic rate ($P = 0.03$), and so was included in the statistical model for this analysis only. [4] First-pass intestinal synthesis values were calculated, within piglet, by subtracting the intraportal value from the intragastric value. [5] There was no significant difference between the intragastric and intraportal rates of glutamate synthesis of arginine for the +Orn and +α-KG/+Orn diets; therefore the first-pass intestinal rate of synthesis is not significantly different from 0.

enterocytes (7,8,19), and this is the most likely explanation for the significant effect of day on the arginine synthetic rate. There was no effect of diet on either the intraportal or intragastric rates of arginine synthesis from ornithine (**Table 6.3**). For both the intraportal and intragastric rates of glutamate synthesis, piglets in the +α-KG/+Orn group had the greatest rate, and piglets in the +α-KG group had the lowest synthetic rate ($P < 0.05$) (**Table 6.3**), which corresponded to the fact that these two groups had the highest and lowest intragastric glutamate fluxes, respectively. For the intragastric infusion, the rate of glutamate synthesis in the piglets in the basal group was not different from the rates in the +α-KG and +Orn groups. Intragastric glutamate synthesis in the +Orn group was greater than in the +α-KG group ($P < 0.05$), but was not different from the other two groups (**Table 6.3**). For the intraportal rate of glutamate synthesis, piglets in the basal and +α-KG groups had lower rates of synthesis than piglets in the +Orn group ($P < 0.05$), which had a lower rate of synthesis than the piglets in the +α-KG/+Orn group ($P < 0.05$) (**Table 6.3**). Proline synthesis from ornithine was higher in the piglets receiving ornithine in their diets ($P < 0.05$) than in piglets receiving either of the basal and +α-KG diets, regardless of the route of isotope infusion (**Table 6.3**).

First-pass intestinal metabolism played a large role in ornithine conversion to its various metabolites (**Table 6.3**). The rate of arginine synthesis during first-pass intestinal metabolism was greater in piglets receiving the basal and +α-KG diets ($P < 0.05$) than in piglets receiving the +Orn and +α-KG/+Orn diets (**Table 6.3**). There was a tendency for glutamate synthesis during first pass intestinal metabolism to be affected by diet ($P = 0.08$). The rate of glutamate synthesis occurring during first-pass intestinal metabolism in the piglets in the +Orn and +α-KG/+Orn groups was not different from zero, because there were no significant differences between the intragastric and intraportal rates of glutamate synthesis (**Table 6.3**), while there was a significant intestinal conversion in piglets in the +α-KG and basal groups. Proline synthesis from ornithine during first-pass intestinal metabolism was greater in piglets receiving the +Orn and +α-KG/+Orn than in the other two groups of piglets ($P < 0.05$) (**Table 6.3**).

6.4 **Discussion**

6.4.1 *Ornithine α-ketoglutarate versus ornithine as an arginine precursor*

This study is the first, to the best of our knowledge, to use the infusion of isotopes to quantify the effects of OKG on metabolism and assess its effectiveness as an arginine precursor in neonates. Because there is no previous research in neonates relating to the metabolism of OKG, the only basis of comparison for the present results is to literature in adults and in rodents; therefore, these comparisons should be interpreted carefully with the understanding that the piglet is not an experimental model for adult metabolism. The co-administration of ornithine and α-ketoglutarate to neonatal piglets in the present study did not affect whole-body arginine status (**Tables 6.1** and **6.2**) or arginine synthesis from ornithine (**Table 6.3**) compared to piglets receiving ornithine alone. We have previously shown that the addition of an effective arginine precursor, citrulline, to the arginine deficient basal diet resulted in lower plasma ammonia and urea concentrations and a higher plasma arginine concentration and whole-body arginine flux (1). We did not observe any effects on these parameters in the piglets receiving the +α-KG/+Orn diet compared to those receiving the +Orn diet; therefore, we concluded that OKG was no more effective than the molar equivalent of ornithine as an arginine precursor.

One of the fundamental differences between the present study, and the previous study in adult humans (5), is that the piglets in the present study were in a state of chronic arginine deficiency. We have previously shown that the primary limitation in arginine synthesis in neonatal piglets receiving an arginine deficient diet was citrulline formation (1), whereas the proposed mechanism of action of OKG (5,20) in adult humans both assumed and seemed to demonstrate that ornithine availability was the limiting factor for arginine synthesis. For this reason we formulated the objective of comparing ornithine and ornithine + α-ketoglutarate in the neonatal piglet to determine whether this could be a secondary limitation for arginine synthesis in the neonate.

There are several other possible reasons why the proposed mechanism (3,5) whereby OKG may be an effective arginine precursor in adult humans does not appear to be the case in neonatal piglets (5). First, studies in humans showed that a bolus infusion of OKG was necessary to achieve an increase in plasma arginine concentrations (5,20). The authors proposed that a bolus dose was necessary to rapidly saturate the ornithine to α-ketoglutarate pathway, and allow for the diversion of ornithine to arginine synthesis (20). A continuous infusion of arginine was used in the present study to allow steady state isotope kinetics to be used, and this raises the question of whether a bolus dose may be necessary to observe effects on arginine synthesis. In the present study, the rate of OKG infusion was ~1.9 g/(kg·hr), or more than ten times greater than the bolus doses given to humans (~0.15 g/kg) (5,20). This very high infusion rate, representing ~80 % of the intragastric ornithine flux (**Table 6.2**), was used to ensure saturation of the metabolic pathways. Therefore, it is unlikely that the use of a continuous infusion was the reason for the lack of effect of OKG administration on arginine synthesis. Second, the metabolism of OKG may be different in neonatal versus older mammals. However, in both week-old suckling piglets and 58-day old weaned piglets, the activity of OAT is substantially higher in the direction of P5C versus ornithine formation (19,21); therefore, this does not support OKG being used more effectively as an arginine precursor in weaned piglets than in suckling piglets. Finally, the addition of OKG in the adult studies was in the form of a neutral ionic salt (5), whereas in the present study three acidic molecules (two ornithine-HCl and one α-ketoglutaric acid molecules) were used. However, because OKG is an ionic salt it readily dissociates in solution. Both diets still contained the same molar proportions of the ornithine and α-ketoglutarate and therefore the only difference would have been the pH of the diet. Because the diet was infused enterally into the acidic environment of the stomach, the lower pH of the mixture compared to the salt would not have affected the ability of the +α-KG/+Orn to act as an arginine precursor.

6.4.2 *Ornithine metabolism and the effects of first-pass intestinal metabolism*

The following stochastic model was used for both the intragastric and intraportal routes of ornithine infusion to calculate the amount of *de novo* ornithine synthesis and non-oxidative ornithine disposal (**Table 6.4**):

Ornithine flux = ornithine intake + *de novo* ornithine synthesis
= ornithine oxidation + non-oxidative ornithine disposal.

Because ornithine is not a component of protein, ornithine use for protein synthesis and ornithine entering the plasma pool from protein breakdown, were not included in this model. The difference between the intragastric and intraportal values represents the contribution of first-pass intestinal metabolism.

The whole-body (intragastric) metabolism of ornithine differed among the groups; the addition of ornithine, as either ornithine-HCl or as ornithine-HCl + α-ketoglutaric acid, to an arginine-deficient diet resulted in a 43-63% decrease in *de novo* ornithine synthesis, a 3-fold increase in oxidative ornithine disposal, and a ~70% increase

Table 6.4 A stochastic model of the effect of first-pass intestinal metabolism on ornithine metabolism in piglets enterally-fed either the basal diet or the basal diet supplemented with α-ketoglutarate, ornithine, or both[1]

	Diet			
	Basal	**+α-KG**	**+Orn**	**+ α-KG/ +Orn**
Ornithine entry into the plasma pool				
			μmol/(kg·h)	
Dietary intake				
intragastric infusion	0	0	383	393
intraportal infusion[2]	0	0	322	330
De novo synthesis				
intragastric infusion	234	241	90	134
intraportal infusion	126	105	78	79
Ornithine removal from the plasma pool				
			μmol/(kg·h)	
Oxidation[3]				
intragastric infusion	46	37	142	179
intraportal infusion	45	36	156	176
Non-oxidative disposal[4]				
intragastric infusion	188	204	331	348
intraportal infusion	81	69	244	233

[1] For both routes of infusion, ornithine flux (**Table 6.2**) was equivalent to ornithine intake + de novo ornithine synthesis, or to ornithine oxidation + non-oxidative ornithine disposal. [2] For the intraportal ornithine metabolism, the first-pass intestinal ornithine extraction value (**Table 6.2**) was multiplied by ornithine intake to calculate the amount of ornithine extracted during first-pass intestinal metabolism, so that the intraportal arginine intake could be calculated by subtracting first-pass intestinal extraction from dietary intake. [3] The molar rate of ornithine oxidation was calculated for both the intraportal and intragastric routes of ornithine infusion by multiplying the percent of ornithine dose oxidized (**Table 6.2**) by the respective ornithine flux (**Table 6.2**). [4] Non-oxidative ornithine disposal represents the conversion of ornithine to other metabolites including arginine, glutamate and proline.

in non-oxidative ornithine disposal (**Table 6.4**). Similar to arginine intake regulating arginine synthesis in piglets (10,12), ornithine intake also regulated ornithine synthesis, with increasing intake resulting in decreased synthesis. To the best of our knowledge, the effects of ornithine intake on ornithine oxidation have not been previously studied *in vivo* in neonatal piglets; however, studies in adult males showed that ornithine oxidation increases with ornithine flux (22,23). Therefore, similar to adult humans, ornithine oxidation in neonatal piglets appears to be regulated, at least in part, by ornithine intake or status. There was an increase in whole-body proline synthesis as a result of ornithine supplementation (**Table 6.3**), which agrees with results from previous studies in humans and rodents, where proline concentrations in the plasma (5,20,24-26) and tissues (24) were consistently higher in the subjects receiving OKG versus an ornithine-free solution.

First-pass intestinal metabolism is a major site of the regulation of ornithine metabolism in response to ornithine intake. For piglets in the basal and +α-KG groups, where *de novo* ornithine synthesis was the greatest, first-pass intestinal metabolism accounted for more than half of whole-body ornithine synthesis (**Table 6.4**). This confirms previous *in vivo* tracer research that found of first-pass intestinal metabolism was the primary site of ornithine formation in enterally-fed piglets (9). Unlike ornithine synthesis, however, there was little difference between the intragastric and intraportal rates of ornithine oxidation, regardless of ornithine intake, indicating that first-pass intestinal metabolism made little contribution to ornithine oxidation (**Table 6.4**). This is in agreement with *in vitro* data which found that in week-old piglet enterocytes, ornithine oxidation was negligible (27).

For all four groups of piglets, approximately 100 μmol/(kg·h) of ornithine was converted to other metabolites during first-pass intestinal metabolism (**Table 6.4**); however, the metabolite products differed depending on the diet (**Table 6.3**). Piglets receiving the basal and +α-KG diets synthesized more arginine and glutamate than the piglets receiving the +Orn and + α-KG/+Orn diets, who synthesized more proline during first-pass intestinal metabolism. One of our previous isotopic studies in neonatal piglets showed that proline synthesis from ornithine is dependent on small intestinal metabolism (9), and the results from the present study further demonstrate the importance of small intestinal metabolism for proline synthesis. Although whole body (intragastric) rates of arginine and glutamate synthesis from ornithine were quite constant between diets, the intraportal conversion rates were significantly lower in the piglets receiving the diets without ornithine (**Table 6.3**), resulting in a greater calculated rate of first-pass intestinal synthesis. Therefore, when the ornithine tracer bypassed first pass intestinal metabolism, in the arginine-deficient piglets not receiving dietary ornithine, other tissues, possibly the liver, extracted more ornithine tracer without a resulting increase in arginine or glutamate release than when ornithine was included in the diet. The first-pass hepatic extraction of ornithine was not determined in the present study, but a study using perfused rat livers found that there was hepatic uptake of ornithine that was not affected by a-ketoglutarate addition to the perfusate (28). The hepatic metabolism of ornithine and arginine is complex (28), and additional research is necessary to quantify *in vivo* the relationship between the hepatic metabolism of these amino acids and their dietary intake.

6.4.4 *Conclusion.*

The primary objective of the present study was to determine whether OKG was a more effective arginine precursor than ornithine. Based on whole-body arginine status and arginine synthesis from ornithine, OKG was no better than ornithine alone as an arginine precursor. Furthermore, ornithine administration in general did not affect arginine metabolism differently than feeding diets devoid of ornithine. This supports the data of our previous study which found that the limitation in arginine synthesis was citrulline formation (1). Ornithine, but not α-ketoglutarate, intake had profound effects on ornithine metabolism, by decreasing de novo synthesis and increasing whole-body oxidative and non-oxidative ornithine disposal. First-pass intestinal metabolism was important for ornithine synthesis and non-oxidative ornithine disposal, but not for ornithine oxidation. Arginine is an important amino acid for neonatal health and survival (29-31), and although further research is still required to complete our understanding of its endogenous synthesis, we now have a better understanding of ornithine metabolism, which is one of the major intermediates in *de novo* arginine synthesis.

6.5 Literature cited

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7.0 A MULTI-TRACER STABLE ISOTOPE APPROACH TO STUDYING THE EFFECTS OF ARGININE INTAKE ON WHOLE-BODY ARGININE METABOLISM IN ENTERALLY-FED PIGLETS

7.1 Introduction

In previous studies examining endogenous arginine synthesis in neonatal piglets (1-3), we used the proline to arginine conversion as the measure of whole-body arginine synthesis, because in a study where both proline and glutamate radioisotopes were intragastrically administered, the only detectable transfer of radioactivity was from proline to arginine (1). Therefore, it was concluded that glutamate, specifically dietary glutamate, was not a precursor for endogenous arginine synthesis. We have previously shown that ~40-60% of whole-body arginine synthesis from proline occurs during the peripheral metabolism of circulating proline (2); therefore, it is possible that circulating glutamine/glutamate may be used in arginine synthesis as has been suggested by others (4). Dietary glutamate is extensively oxidized during first-pass splanchnic metabolism in piglets (5); therefore, if the majority of the intragastrically-administered glutamate isotope was oxidized before reaching general circulation, then the previous study (1) would not have detected the possible contribution of circulating glutamate to whole-body arginine synthesis. This would have underestimated the possible contribution of glutamate to whole-body arginine synthesis.

Endogenous arginine synthesis in adult humans (6) has been measured using conversion of citrulline to arginine. Although the 5-carbon backbone of citrulline is derived from ornithine, which can originate from either proline or glutamine/glutamate via pyrroline-5-carboxylate (P5C) or from arginine which is converted to ornithine via the action of arginase, all the carbon that appears in arginine that is endogenously synthesized must first be converted to citrulline. Therefore, the conversion of citrulline to arginine is the best measure of total endogenous arginine synthesis. By comparing the rate of conversion of proline to arginine to the rate of the citrulline to arginine conversion, then the relative importance of proline as an arginine precursor can be assessed, and subsequently the relative contributions of other arginine precursors can also be determined.

In a series of previous studies, we measured the fractional net conversion of proline to other metabolites such as citrulline and ornithine (1,2,7); however, because neither citrulline nor ornithine flux were measured, the molar rates of conversions between metabolites could not be calculated. If the molar rates of conversion between the major intermediates in the proline to arginine synthetic pathway, specifically proline, ornithine, citrulline and arginine, are known, then it will be possible to identify the steps in this conversion that are limiting to whole-body arginine synthesis. In a previous study, we found that the addition of either arginine or citrulline, but neither ornithine nor proline, to an arginine-deficient basal diet resulted in similar improvements in whole-body arginine status (3). These findings indicated that it was citrulline formation that was limiting to endogenous arginine synthesis; however, rates of isotopic conversion were not measured in the previous study (3).

The effect of arginine intake on the rate of whole-body nitric oxide synthesis has not been previously measured in neonates. In a previous study in enterally-fed neonatal piglets, arginine intake tended to have an effect on plasma nitric oxide concentrations, with piglets receiving a generous intake of arginine having numerically higher concentrations than those receiving a deficient diet (1). In adult men, arginine intake did not have an effect on nitric oxide synthesis (8,9); however, the dietary regulation of arginine synthesis is very different in neonates versus adults (1,6). Therefore, it is possible that the response of nitric oxide synthesis to different arginine intakes is also different in neonates versus adults. The effect of arginine intake on whole-body nitric oxide synthesis in neonates is of particular interest, because low plasma arginine concentrations (10) and fluxes (11) have been described in infants suffering from persistent pulmonary hypertension of the neonate (PPHN) (11) and necrotizing enterocolitis (NEC) (10). For both PPHN and NEC, arginine supplementation has been shown to reduce the incidence (12,13) of the disease or reduce the severity of the symptoms (14), and for both pathologies the role of arginine as a nitric oxide precursor has been suggested as a possible reason for the protective effect of arginine.

The major objectives in this study were to: 1) determine the portion of total arginine synthesis originating from proline, and the effect of dietary arginine intake on the contribution of proline to total arginine synthesis; 2) measure the rates of conversion between the various intermediates in the arginine synthetic pathway and determine the effect of diet on these conversions; 3) obtain isotopic confirmation for the limiting step in endogenous arginine synthesis; and 4) determine the effect of dietary arginine intake on whole-body nitric oxide synthesis in enterally-fed neonatal piglets. In order to address these four major objectives, the present study was designed to use the simultaneous infusion of four isotope tracers: [guanido-$^{15}N_2$]arginine, [ureido-^{13}C; 5,5-$^{2}H_2$]citrulline, [U-$^{13}C_5$]ornithine and [^{15}N, U-$^{13}C_5$]proline, in individual piglets receiving either a deficient or generous intake of arginine. This study also marks the first time that we have used stable isotopes to study endogenous arginine synthesis in neonatal piglets and therefore comparisons will be made among similar diets from previous radioisotope tracer experiments (2,3,15).

7.2 Materials and methods

7.2.1 *Animals and surgical procedures*

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee for the University of Alberta. Ten intact male Landrace/ Large White/ Duroc piglets (Hypor, Regina SK, Canada) (1.4 – 1.8 kg) were obtained from the University of Alberta Swine Research and Technology Centre at 1-2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant a gastric catheter for diet and isotope infusion and a femoral vein catheter for blood sampling. Pre-operative procedures, surgical procedures, post-surgical care and piglet housing were as previously described (2,15).

7.2.2 *Diets and treatment groups*

The complete elemental diet was designed to meet all nutrient requirements of piglets (16), with targeted nutrient intakes of 15 g amino acid/(kg·d), and 1.1 MJ metabolizable energy/(kg·d), with glucose and lipid (Intralipid 20%; Fresenius Kabi AB, Bad Homburg, Germany) each providing 50% of the non-protein energy intake. The diet was continuously infused enterally via the gastric catheter using pressure-sensitive infusion pumps. Diet composition (2,3), preparation (17), and piglet adaptation to the enteral diet (2,3) were all as previously described.

Piglets received the complete diet until the morning of d 3 and then were randomly assigned (n = 5/diet) to either a generous [+Arg, 1.80 g/(kg·d)] or deficient [-

Arg, 0.20 g/(kg·d)] intake of arginine for the remainder of the trial. The amino acid compositions of these diets were identical to the basal and +Arg diets that were recently described (3). These diets were chosen to represent diets that would result in minimum and maximum rates of arginine synthesis (1) without creating adverse metabolic effects (18). Diets were made isonitrogenous by adjusting the concentrations of alanine and glycine (3).

7.2.3 *Blood sampling*

Blood samples (2 mL) were collected every 24 hours, beginning immediately prior to the allocation to test diets on d 3 and continuing until the end of the trial. The daily blood samples were used for the determination of plasma ammonia, urea nitrogen and nitric oxide concentrations, and the blood sample taken on d 7 was also used for the determination of plasma amino acid concentrations. As described under *constant tracer infusions*, additional blood samples were taken during the tracer infusion on d 7.

7.2.4 *Constant tracer infusions*

On the morning of d 7, all piglets received a primed constant infusion of four stable isotopes: L-[guanido-$^{15}N_2$]arginine-HCl [m+2; prime: 12 μmol/kg; constant: 20 μmol/(kg·h)] (98% ^{15}N; Cambridge Isotope Laboratories, Andover MA, USA), L-[ureido-^{13}C; 5,5-$^{2}H_2$]citrulline [m+3; prime: 5 μmol/kg; constant: 5 μmol/(kg·h)] (99% ^{13}C, 98% ^{2}H; Cambridge Isotope Laboratories, Andover MA, USA), L-[U-$^{13}C_5$]ornithine-HCl [m+5; prime: 13 μmol/kg; constant: 10 μmol/(kg·h)] (98% ^{13}C; Cambridge Isotope Laboratories, Andover MA, USA), and L-[^{15}N; U-$^{13}C_5$]proline [m+6;

prime: 20 µmol/kg; constant: 40 µmol/(kg·h)] (98% ^{15}N , 98% ^{13}C ; Cambridge Isotope Laboratories, Andover MA, USA). The constant infusion lasted 6 hours and blood (1 mL) was sampled at –60, -30, 0 (3 baseline samples), 60, 120, 180, 240, 270, 300, 330 and 360 min.

For one piglet in each treatment group, the prime and constant infusion rates of the arginine [6 µmol/kg; constant: 10 µmol/(kg·h)] and proline [20 µmol/kg; constant: 10 µmol/(kg·h)] isotopes were lower than in the remaining 4 piglets in each group. The infusion rates were increased as a precaution to ensure that there would be adequate label transfer between intermediates, and that potentially small differences between treatment groups could be detected. However, even in the two piglets receiving the lower rates of arginine and proline infusion there was measurable isotopic enrichment in all of the product amino acids studied. In the case of the proline, ornithine and arginine isotopes, the prime to constant ratio was based on previously validated rates in the piglet model (1,7). The prime to constant ratio used for the citrulline isotope was based on the ratio previously used in human studies (6,9,19). The constant infusion rates in the present study were higher than those previously used in weaned pigs and adult humans (6,20-22), where the isotope was infused intravenously, to account for the possible splanchnic extraction of the intragastrically-administered isotope, as has been described for arginine and ornithine (3,15,23). However, the infusion rates of 5 – 20 µmol/(kg·h) were within the range that has been previously used for other amino acid isotopes in neonatal infants (11,24,25). The four isotopes used in the present study were selected based on the fact that they each produced different isotopomers for each of the amino acids studied (arginine, citrulline, ornithine and proline) (**Table 7.1**); therefore, with a single isotopic infusion, we could measure the metabolism and inter-conversions of all four amino acids.

To make the isotope infusion solution, each of the isotopes was separately dissolved in sterile water, to give a concentration of twice the hourly infusion rate per mL, and then filtered through a 0.22 μm Millipore filter (Millipore, Bedford MA, USA). Immediately prior to the constant infusion, the four isotope solutions were mixed together in a 1:1:1:1 ratio; therefore, each piglet received 2 mL/(kg·h) of infusion solution during the constant infusion.

At the end of the d 7 infusion, piglets were euthanized with an injection of 500 mg of pentobarbital sodium (Euthansol, 340 g/L; Schering Canada Inc., Pointe Claire PQ) into the femoral vein catheter.

7.2.5 *Analytical procedures*

The d 7 plasma amino acid concentrations were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (26,27).

Plasma ammonia (Reference 200-02; Diagnostic Chemical Limited, Charlottetown PEI, Canada), urea nitrogen (Sigma Procedure No. 640; Sigma Diagnostics, St. Louis MO, USA) and nitric oxide (Product DINO-250; Bio Assay Systems, Hayward CA, USA) concentrations were determined every 24 h during test diet infusion (d 3 – d 7) using spectrophotometric assays.

For the plasma samples taken during the d 7 constant infusion, the isotopic enrichment of the each of the infused isotopomers and its product amino acids were

Table 7.1 Arginine, citrulline, ornithine and proline isotopomers derived from each of the infused isotopes

	Product isotopomer			
Infused isotope	**Arginine**	**Citrulline**	**Ornithine**	**Proline**
L-[guanido-$^{15}N_2$]arginine	L-[guanido-$^{15}N_2$]arginine	L-[ureido-^{15}N] citrulline	Not measured	Not measured
L-[ureido-^{13}C; 5,5-$^{2}H_2$] citrulline	L-[guanido-^{13}C; 5,5-$^{2}H_2$] arginine	L-[ureido-^{13}C; 5,5-$^{2}H_2$] citrulline	L-[5,5-$^{2}H_2$] ornithine	L-[5-^{2}H] proline
L-[U-$^{13}C_5$] ornithine	L-[1,2,3,4,5-$^{13}C_5$]arginine	L-[1,2,3,4,5-$^{13}C_5$]citrulline	L-[U-$^{13}C_5$] ornithine	L-[U-$^{13}C_5$] proline
L-[^{15}N, U-$^{13}C_5$] proline	L-[^{15}N, 1,2,3,4,5-$^{13}C_5$] arginine	L-[^{15}N, 1,2,3,4,5-$^{13}C_5$] citrulline	L-[^{15}N, U-$^{13}C_5$] ornithine	L-[^{15}N, U-$^{13}C_5$]proline

measured using liquid chromatography-tandem mass spectrometry (LC-MS). The product amino acids for each of the infused isotopes were as described in **Table 7.1**.

To prepare the samples for LC-MS analysis, 25 μL of plasma was deproteinized by adding 500 μL of methanol, vortexed for 30 seconds and then centrifuged at 9,000 X g for 10 minutes at 37°C. The supernatant was transferred to derivatizing tubes and dried under nitrogen at 37 °C. The dried samples were derivatized with 100 μL of 3.0N hydrochloric acid-butanol derivative reagent (Regis Technologies Inc, Morton Grove, IL), at 55°C for 20 minutes, and dried under nitrogen at 37 °C. The dried amino acids were reconstituted in 250 μL of 0.1% formic acid.

An API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX) operated in positive ionization mode with the TurboIonSpray ionization probe source (operated at 5800 V and 600 °C), coupled to an Agilent 1100 HPLC system (Agilent Technologies Canada Inc, Mississauga, Canada) was used. All aspects of system operation and data acquisition were controlled using The Analyst NT v1.4.1 software.

Maximum sensitivity for the butylated labeled and unlabelled isotopes and their isotopomers were achieved by measuring specific transitions of each isotope and its isotopomers to its product ions resulting from the fragmentation of the protonated $[M+H]^+$ molecules. The detection of unlabelled proline, ornithine, citrulline, and arginine were made by employing their characteristic transitions of butylated-parent to daughter ions at m/z 172.2 to 70, 189.3 to 70, 232.3 to 70 and 231.3 to 70, respectively. Similar characteristic transitions were applied to their enriched isotopomer distributions. Prior to entering the triple quadrupole mass spectrometer, the individual amino acids were separated using a Dionex Acclaim organic acid column (5µm 120A, 4.0 x 250 mm; Dionex Canada Ltd, Oakville ON) and were eluted over 15 minutes with a binary liquid chromatography gradient (0-30% acetonitrile, containing 0.1% formic acid) at a flow rate of 700 µL/min. The retention times were ~ 3.75, 5.93, 8.06, and 9.14 minutes for ornithine, arginine, citrulline, and proline, respectively.

7.2.6 *Calculations*

For each isotopomer of each amino acid studied, the mean ratio of the peak area of the labeled amino acid to the peak area of the unlabeled amino acid, for both baseline and plateau samples, was used to calculated the enrichment using the following formula:

$$\text{Enrichment} = ([\text{ratio}_{\text{plateau}} - \text{ratio}_{\text{baseline}}]/[1 + (\text{ratio}_{\text{plateau}} - \text{ratio}_{\text{baseline}})]) \text{ X } 100\%$$

For the infused isotopomers, the plateau included at least 4 measurements, having a CV of less than 10%, and for the product isotopomers, each plateau included at least 3 points with a CV of less than 15%. The enrichment values were then used to calculate the whole-body flux for each of the infused amino acids and the conversion rate of each of the infused amino acids to its product amino acids. Flux (Q) was calculated by:

$$Q\ [\mu\text{mol}/(\text{kg}\cdot\text{h})] = i \text{ X } [(E_i/E_p) - 1],$$

where i was the rate of isotope infusion [in µmol/(kg·h)], E_i was the enrichment of infused isotope and E_p was the plasma enrichment calculated using the formula above. Flux includes the amount of the amino acid entering the plasma pool through the diet, *de novo* synthesis and protein breakdown, or leaving the pool through protein synthesis, oxidation or conversion to other metabolites.

The molar rates of conversion ($Q_{precursor \rightarrow product}$) for each infused amino acid to its product amino acids were calculated using:

$Q_{precursor \rightarrow product}$ [μmol/(kg·h)] = ($\text{Enrichment}_{product\ amino\ acid}/\text{Enrichment}_{precursor\ amino\ acid}$) X $Q_{product\ amino\ acid}$ X [$Q_{precursor\ amino\ acid}$ / ($i_{precursor\ amino\ acid}$ + $Q_{precursor\ amino\ acid}$)]

The term [$Q_{precursor\ amino\ acid}$ / ($i_{precursor\ amino\ acid}$ + $Q_{precursor\ amino\ acid}$)] was used to correct for the infused tracer contribution to the $Q_{precursor \rightarrow product}$.

The $Q_{arginine \rightarrow citrulline}$ was used as a measure of nitric oxide synthesis, because for every mol of citrulline formed from arginine, a mol of nitric oxide was also formed. This approach to measuring nitric oxide synthesis has been previously used in both humans (19) and pigs (22). The portion of arginine flux that was used for nitric oxide synthesis was calculated using:

% Arginine flux to NO = ($Q_{arginine \rightarrow citrulline}$/ $Q_{arginine}$) X 100%.

The relative importance of proline as an arginine precursor was calculated by comparing the $Q_{proline \rightarrow arginine}$ with the $Q_{citrulline \rightarrow arginine}$, using the following formula:

% total arginine synthesis from proline = ($Q_{proline \rightarrow arginine}$/ $Q_{citrulline \rightarrow arginine}$) X 100%.

7.2.7 *Statistical analysis*

Unless specifically noted, all data were analyzed using the mixed model of SAS Version 9.1 (SAS Institute, Cary NC, USA), and data were considered statistically significant if $P < 0.05$, and trends were considered at $0.05 < P < 0.10$. When the fixed effects were significant ($P < 0.05$), least squares means were compared using the pdiff test.

The dependent variables plasma ammonia, urea nitrogen and nitric oxide concentrations were analyzed using repeated measures analysis where the fixed effect was diet and the random variables were piglet nested within diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion.

The remaining variables, including plasma amino acid concentrations, the fluxes of the infused amino acids, $Q_{\text{product}\rightarrow\text{precursor}}$, the portion of arginine flux converted to nitric oxide and the relative importance of proline as an arginine precursor were analyzed using diet as the fixed effect and piglet nested within diet as the random variable.

7.3 **Results**

7.3.1 *Piglet performance*

All piglets remained healthy and active for the duration of the 7 d trial. Although there were no differences in piglet weight at the initiation of test diet infusion (**Table 7.1**), piglets receiving the –Arg diet gained significantly more weight than piglets receiving the +Arg diet ($P < 0.01$) (**Table 7.2**), resulting in a trend ($P = 0.06$) for a higher body weight in the –Arg piglets at the end of the experiment. A similar effect of arginine intake on body weight has been recently described in enterally-fed neonatal piglets (1), and was attributed to an increase in extracellular fluid volume due to the dietary arginine deficiency and subsequent large increase in plasma glutamine concentrations. House et al. (28) observed that piglets receiving a parenteral solution supplemented with glutamine gained more weight than piglets receiving a glutamine-free solution, and the weight differences in weight gain were largely attributed to an increase in total body water. Although body composition was not measured in this study, the explanation offered by Wilkinson et al (1) for differences in weight gain due to arginine intake seems to be a plausible explanation for the present findings, and is also supported by the –Arg piglets in the present study having 5-fold higher plasma glutamine concentrations (**Table 7.3**) than the +Arg piglets.

7.3.2 *Plasma amino acid concentrations*

Plasma arginine and ornithine concentrations were 9.7 and 2.2-fold greater, respectively, in the piglets receiving the +Arg than in piglets receiving the –Arg diet (P <

Table 7.2 Performance of enterally-fed neonatal piglets receiving either the +Arg [1.80 g/(kg·d)] or -Arg [0.20 g/(kg·d)] diet[1]

	+Arg diet	-Arg diet	Pooled SE	p-value
Weight at initiation of test diet (Day 3) (kg)	1.87	1.89	0.05	NS[2]
Weight gain- test diet (Day 3-7) (kg)	0.64	0.82	0.04	< 0.01
Rate of weight gain- test diet (Day 3-7) [g/(kg·d)]	87	109	4	< 0.01
Final weight (Day 7) (kg)	2.51	2.71	0.07	0.05 < P < 0.10

[1] Values are least squares means, n = 5. [2] Not significant (P > 0.10).

Table 7.3 Day 7 plasma amino acid concentrations (μmol/L) in enterally-fed neonatal piglets receiving either the +Arg [1.80 g/(kg·d)] or –Arg [0.20 g/(kg·d)] diet[1]

Amino acid	Diet +Arg	 -Arg	Pooled SE	P-value
Indispensable				
Histidine	45	101	13	< 0.05
Isoleucine	113	164	24	NS[2]
Leucine	298	249	27	NS
Lysine	312	350	44	NS
Methionine	96	53	6	< 0.001
Phenylalanine	82	92	5	NS
Threonine	860	468	61	< 0.01
Tryptophan	19	20	2	NS
Valine	250	315	17	< 0.05
Conditionally Indispensable				
Arginine	267	25	22	<0.0001
Cystine	10	12	2	NS
Glutamine	69	419	35	< 0.0001
Glycine	1442	2917	304	< 0.01
Proline	466	474	54	NS
Taurine	131	141	9	NS
Tyrosine	69	104	10	< 0.05
Dispensable				
Alanine	397	843	62	< 0.001
Aspartate	9	10	1	NS
Citrulline	120	118	16	NS
Cystathione	93	62	4	<0.001
Glutamate	83	77	6	NS
Hydroxyproline	104	86	7	NS
Ornithine	132	41	6	< 0.0001
Serine	347	665	60	< 0.01

[1] Values are least squares means, n = 5. [2] Not significant (P > 0.10).

0.05, **Table 7.3**). Plasma histidine, valine and tyrosine concentrations were higher in the –Arg piglets than the +Arg piglets ($P < 0.05$, **Table 7.3**). The higher plasma concentrations of these indispensable and conditionally indispensable amino acids are believed to be due to a reduction in protein synthesis due to the arginine deficiency. We have previously observed that the plasma concentrations of indispensable amino acids are higher in piglets receiving a diet limiting in another of the indispensable amino acids (3,29). Glycine and alanine concentrations were higher in the –Arg diet, in order to make the two diets isonitrogenous, which explains why the plasma concentrations of these amino acids were also higher in the –Arg piglets ($P < 0.05$, **Table 7.3**). Piglets in the –Arg group had higher plasma serine and lower plasma methionine, cystathione and threonine concentrations than piglets in the +Arg group ($P < 0.05$, **Table 7.3**). These differences in plasma serine, methionine and threonine concentrations have been previously observed (1-3), and because glycine is involved in the metabolic pathways associated with threonine and serine metabolism, and serine is an intermediate in the metabolism of methionine to cysteine, differences between diets in the plasma concentrations of these amino acids are believed to be related to the different glycine intakes, although direct experimental confirmation of this is necessary.

7.3.3 *Plasma ammonia, urea and nitric oxide concentrations*

We have recently discussed the use of plasma ammonia and urea concentrations as indicators of whole-body arginine status (3), although a similar relationship between arginine status and plasma nitric oxide concentrations has not been previously established. Diet ($P = 0.0003$) and day ($P < 0.0001$), but not their interaction ($P > 0.05$)

had a significant effect on plasma ammonia concentrations, and from d 5 onwards, piglets in the –Arg group had higher plasma ammonia concentrations than those in the +Arg group (**Table 7.4**). Plasma urea concentrations were affected by diet ($P = 0.0018$), day ($P = 0.0026$) and their interaction ($P = 0.0282$) (**Table 7.4**). Although the plasma urea concentration in the +Arg piglets remained unchanged throughout the 5 d period, the urea concentrations in the –Arg piglets increased until d 5 and then remained constant, and higher than in the +Arg piglets, for the remainder of the trial (**Table 7.4**). Plasma nitric oxide remained unchanged regardless of diet, day or the interaction between diet and day (**Table 7.4**).

7.3.4 *Intragastric amino acid fluxes*

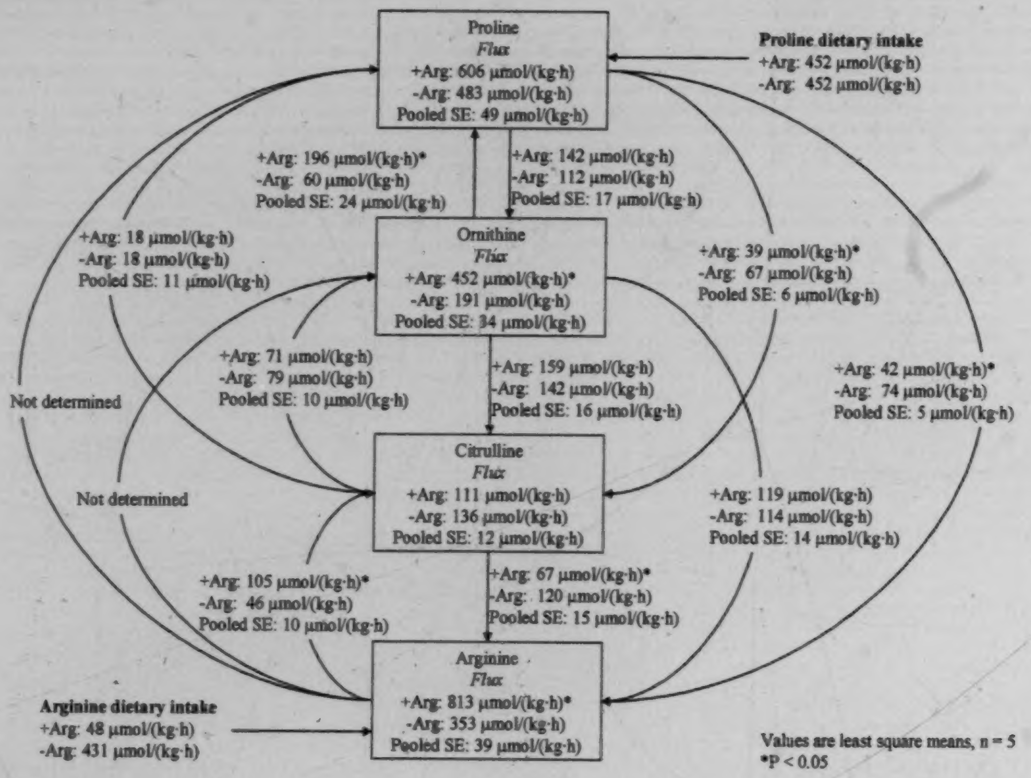
Because the isotopes were administered intragastrically, the fluxes were influenced by both first-pass splanchnic metabolism and the metabolism by other peripheral tissues (such as muscle, kidney, lung, and intestinal metabolism of arterial substrates). Whole-body arginine and ornithine fluxes were 130% and 137% greater ($P < 0.05$), respectively, in the piglets receiving the +Arg diet than in those receiving the –Arg diet (**Figure 7.1**). The proline isotope was not as pure as anticipated, having approximately 3.7% of its label as an m+5 proline; however, based on the rate of conversion of m+6 proline to m+6 ornithine, this slight impurity would only have contributed ~1.6 – 1.8% of the measured m+5 ornithine flux [+Arg: 452 μmol/(kg·h); -Arg: 191 μmol/(kg·h)] and because this contribution was well below the standard error [34 μmol/(kg·h)] associated with the m+5 ornithine flux, the contribution of the m+5

Table 7.4 Plasma ammonia (μmol/L), urea (mmol/L) and nitric oxide (μmol/L) concentrations in enterally-fed neonatal piglets receiving either the +Arg [1.80 g/(kg·d)] or –Arg [0.20 g/(kg·d)] diet[1]

	Diet		
Day	**+Arg**	**-Arg**	**Pooled SE**
	Plasma ammonia concentration		
3	33^{a}	44^{a}	9
4	74^{b}	86^{b}	9
5	68^{b}	$101b^{c*}$	9
6	77^{b}	120^{c*}	9
7	88^{b}	122^{c*}	9
	Plasma urea concentration		
3	0.60	0.59^{a}	0.25
4	0.90	$1.59^{b\dagger}$	0.25
5	0.90	2.30^{c*}	0.25
6	1.06	2.85^{d*}	0.25
7	0.78	2.55^{cd*}	0.25
	Plasma nitric oxide concentration		
3	61	69	20
4	62	96	20
5	62	70	20
6	69	89	20
7	63	66	20

[1] Values are least square means, n = 5. Means in a column without a common superscript letter differ, $P < 0.05$. *generous arginine diet value is significantly different from the deficient arginine value ($P < 0.05$), †trend for a dietary effect ($0.05 < P < 0.10$).

Figure 7.1 Schematic summary of the arginine, citrulline, ornithine and proline kinetics in enterally-fed piglets receiving either the +Arg [1.80 g/(kg·d)] or -Arg [0.20 g/(kg·d)] diet[1,2]


Proline
Flux
+Arg: 606 µmol/(kg·h)
-Arg: 483 µmol/(kg·h)
Pooled SE: 49 µmol/(kg·h)
Proline dietary intake
+Arg: 452 µmol/(kg·h)
-Arg: 452 µmol/(kg·h)
+Arg: 196 µmol/(kg·h)*
-Arg: 60 µmol/(kg·h)
Pooled SE: 24 µmol/(kg·h)
+Arg: 142 µmol/(kg·h)
-Arg: 112 µmol/(kg·h)
Pooled SE: 17 µmol/(kg·h)
+Arg: 18 µmol/(kg·h)
-Arg: 18 µmol/(kg·h)
Pooled SE: 11 µmol/(kg·h)
Ornithine
Flux
+Arg: 452 µmol/(kg·h)*
-Arg: 191 µmol/(kg·h)
Pooled SE: 34 µmol/(kg·h)
+Arg: 39 µmol/(kg·h)*
-Arg: 67 µmol/(kg·h)
Pooled SE: 6 µmol/(kg·h)
+Arg: 71 µmol/(kg·h)
-Arg: 79 µmol/(kg·h)
Pooled SE: 10 µmol/(kg·h)
+Arg: 159 µmol/(kg·h)
-Arg: 142 µmol/(kg·h)
Pooled SE: 16 µmol/(kg·h)
Not determined
+Arg: 42 µmol/(kg·h)*
-Arg: 74 µmol/(kg·h)
Pooled SE: 5 µmol/(kg·h)
Not determined
Citrulline
Flux
+Arg: 111 µmol/(kg·h)
-Arg: 136 µmol/(kg·h)
Pooled SE: 12 µmol/(kg·h)
+Arg: 119 µmol/(kg·h)
-Arg: 114 µmol/(kg·h)
Pooled SE: 14 µmol/(kg·h)
+Arg: 105 µmol/(kg·h)*
-Arg: 46 µmol/(kg·h)
Pooled SE: 10 µmol/(kg·h)
+Arg: 67 µmol/(kg·h)*
-Arg: 120 µmol/(kg·h)
Pooled SE: 15 µmol/(kg·h)
Arginine
Flux
+Arg: 813 µmol/(kg·h)*
-Arg: 353 µmol/(kg·h)
Pooled SE: 39 µmol/(kg·h)
Arginine dietary intake
+Arg: 48 µmol/(kg·h)
-Arg: 431 µmol/(kg·h)
Values are least square means, n = 5
*P < 0.05

[1] Values are least squares means, n = 5. [2] All conversion values were calculated by $(\text{Enrichment}_{\text{product amino acid}}/\text{Enrichment}_{\text{precursor amino acid}}) \text{ X } Q_{\text{product amino acid}} \text{ X } [Q_{\text{precursor amino acid}} / (i_{\text{precursor amino acid}} + Q_{\text{precursor amino acid}})]$.

proline to m+5 ornithine flux was considered negligible. Neither proline flux nor citrulline flux was different between treatment groups (**Figure 7.1**).

7.3.5 *Arginine conversion to nitric oxide*

The rate of arginine to citrulline conversion, which is an indirect measure of nitric oxide synthesis, was 228% greater in piglets receiving the +Arg versus –Arg diet ($P < 0.05$) (**Figure 7.1**). However, regardless of arginine intake, the portion of arginine flux converted to nitric oxide was not different (+Arg: 13.1%; -Arg: 12.7%; pooled SE: 2.2%; $P > 0.05$).

7.3.6 *Arginine synthesis from precursor amino acids*

Ornithine conversion to arginine was not affected by arginine intake ($P > 0.05$) (**Figure 7.1**). However, arginine synthesis from both proline and citrulline was greater in the piglets in the –Arg group than in those in the +Arg group ($P < 0.05$) (**Figure 7.1**). In both groups, the portion of total arginine synthesis, which was equivalent to the $Q_{\mathrm{citrulline} \rightarrow \mathrm{arginine}}$, that was accounted for by proline as the arginine precursor, was approximately the same in the two groups (+Arg: 63%; -Arg: 56%; pooled SE: 5%; $P > 0.05$).

7.3.7 *Rates of conversions between other amino acids*

The rate of proline conversion to citrulline was also greater in piglets receiving the –Arg diet ($P < 0.05$) (**Figure 7.1**), and these values were strikingly similar to the rates of proline conversion to arginine for both diets. Piglets receiving the +Arg diet had a higher rate of ornithine conversion to proline than those receiving the –Arg diet ($P < 0.05$) (**Figure 7.1**). None of the other amino acid conversion rates were affected by arginine intake ($P > 0.05$) (**Figure 7.1**).

7.4 Discussion

7.4.1 *Comparison to earlier work with radioisotopes*

This study is unique in that it is the first to use the intragastric infusion of proline, arginine, ornithine and citrulline stable isotopes, in any age of species, to study several aspects of whole-body arginine metabolism simultaneously. The proline, arginine and ornithine fluxes, and the rates of proline and ornithine conversion to arginine have been previously measured in other studies using radioisotopes (1-3,15): the flux of intragastrically-administered proline ranged from 394 – 594 μmol/(kg·h), regardless of arginine intake (1,3); intragastric arginine flux ranged from 661 – 1011 μmol/(kg·h) in piglets receiving a generous arginine diet, and from 290 – 590 μmol/(kg·h) in piglets receiving a deficient arginine diet (1,3,15); the conversion rate of proline to arginine was 40 and 75 μmol/(kg·h) for piglets receiving the +Arg and –Arg diets respectively; and finally, the intragastric ornithine flux and rate of ornithine to arginine conversion in piglets receiving the –Arg diet were 234 and 147 μmol/(kg·h) (15), respectively. The similarities between the radioisotope (1-3,15) and stable isotope data (**Figure 7.1**) are striking, despite the fact that the two sets of values used different analytical techniques. Although the parameters listed above have been measured individually in other studies (1-3,15), these parameters represent only a small amount of the total data reported in the present study. The strength of the present study is that all measurements were made simultaneously and within each piglet; therefore, this study is an extremely comprehensive look at whole-body arginine metabolism in neonatal piglets. Previously unanswered or ambiguous questions with regards to arginine metabolism can now be answered.

7.4.2 *Total arginine synthesis in enterally-fed piglets*

Although previous research showed that the rate of proline conversion to arginine was greater in piglets receiving the -Arg diet than in those receiving the +Arg diet (1,3), this alone does not provide sufficient evidence to unequivocally conclude that whole-body arginine synthesis was increased during arginine deficiency. Nor did it provide the optimal basis of comparison to previous data from adults where the citrulline to arginine conversion rate was used as a measure of arginine synthesis (6). In the previous research (1,3), it was possible that the greater rate of proline to arginine conversion in the –Arg piglets was offset by a decrease in the rate of conversion of other precursors to arginine, resulting in no overall net effect on the total rate of arginine synthesis. For this reason it was necessary to measure the rate of total arginine synthesis, using the rate of citrulline to arginine conversion, to determine the true effect of arginine intake on arginine synthesis.

Total arginine synthesis was greater in piglets receiving a deficient arginine diet than in those receiving a generous intake of arginine (**Figure 7.1**). This is in clear contrast to both adult humans and rats where there was no observed increase in the citrulline to arginine conversion in response to an arginine-free diet (6,30), assessed using the isotopic conversion of citrulline to arginine in the human study (6) and the renal arginine output, determined using the arterio-venous difference method, in the rat study (30). The regulation of arginine synthesis in adult mammals is clearly different than in neonatal mammals; therefore, the findings with regards to arginine metabolism in adults can not be extrapolated to neonates.

In addition to having a greater rate of total arginine synthesis, piglets receiving the –Arg diet also had a greater rate of net arginine synthesis [41 μmol/(kg·h)] than the +Arg piglets [-4 μmol/(kg·h)] (**Figure 7.1**). Net arginine synthesis was calculated by subtracting the rate of citrulline to ornithine conversion, which is the amount of endogenously synthesized arginine that was converted to ornithine and urea via the action of arginase (EC number 3.5.3.1), from the rate of total arginine synthesis. This calculation showed that in piglets receiving a deficient arginine intake, two-thirds of the newly synthesized arginine was available for use in metabolic functions such as protein, creatine, polyamine and nitric oxide synthesis. Piglets receiving the +Arg diet, on the other hand, did not have any net arginine synthesis from citrulline, clearly demonstrating for the first time ~~there~~ is a basal rate of arginine synthesis even when the metabolic arginine requirement (31) is being fully met through dietary intake. However, these data also show that this newly synthesized arginine is converted to ornithine (**Figure 7.1**), which is then likely oxidized to carbon dioxide (15). Therefore, in piglets, similar to humans (21), there appears to be an increase in arginine degradation when dietary arginine intake is in excess of the requirements.

7.4.3 *Relative contribution of proline to total arginine synthesis*

Regardless of arginine intake, proline was the major precursor for arginine synthesis with the proline to arginine conversion accounting for ~60% of total arginine synthesis; therefore, other arginine precursors must account for the remaining 40% of endogenous arginine synthesis, and these other precursors have not been previously identified. *In vitro* enterocyte work has found that although the major precursor for arginine in suckling piglets was proline (32,33), glutamine addition to the incubation medium also increased the arginine content of the cell extracts (33,34), although this work did not distinguish between whether it was the carbon or the nitrogen moiety of the glutamine that was being used for arginine synthesis (34). In piglets receiving either a generous or deficient arginine diet, there was no detectable radioactivity in arginine in response to an intragastric infusion of [3,4-^{3}H]glutamate, and the authors concluded that glutamate was not a precursor for whole-body arginine synthesis (1). However, because glutamate was shown to be extensively catabolized during first-pass splanchnic metabolism (5), only a very small portion of the infused isotope may have reached the general circulation; therefore if circulating glutamate/glutamine was a precursor for arginine synthesis, by either the intestine or other tissues, this contribution may not have been measurable using the intragastric tracer administration. The importance of circulating glutamine and glutamate for whole-body arginine synthesis has not been previously measured in enterally-fed neonatal piglets using intravenously-infused isotopes; however, an arterio-venous study in two-week old suckling piglets found that there was a significant intestinal uptake of glutamine, but not proline, and release of arginine (4).

Another possible precursor for endogenous arginine synthesis is dietary arginine that is hydrolyzed to ornithine. Arginine was extensively converted to ornithine in enterally-fed piglets, particularly in those piglets receiving a generous amount of dietary arginine (2), therefore it is possible that some of this ornithine may have been recycled back to arginine. A study using rat liver perfusions and isolated hepatocytes revealed that extra-mitochondrial arginine was converted extensively to ornithine, presumably via mitochondrial arginase II, and subsequently citrulline, in a series of reactions that appeared to be fairly tightly channeled (35). Although the liver was not a site of endogenous arginine synthesis (2) in piglets, arginase II is located in many tissues including the kidney and intestine (36); therefore, the results from the rat liver perfusion and hepatocyte study (35) may be relevant to arginine metabolism in other tissues. Therefore, arginine itself may be a precursor for arginine synthesis, especially in piglets receiving the +Arg diet where all newly synthesized arginine was shown to be recycled back to ornithine (Section 7.4.2).

7.4.4 *Isotopic evidence that citrulline formation limits endogenous arginine synthesis from proline*

All of the citrulline that was formed from proline was converted to arginine, regardless of arginine intake (**Figure 7.1**), providing strong, direct evidence to confirm that the formation of citrulline limits arginine synthesis in enterally-fed piglets (3). Wu et al (33) proposed that low enterocyte mitochondrial N-acetylglutamate (NAG) concentrations, an essential co-factor of carbamoylphosphate synthetase I (CPS I; EC number 6.3.4.16), may limit citrulline formation in suckling piglets, particularly those older than one week of age (33). To support this proposal, when four-day old suckling piglets were orally-gavaged with N-carbamoylglutamate, a stable NAG analog, twice daily for 10 days, there was an increase in the intestinal formation of both citrulline and arginine *in vitro*, compared to piglets that received a gavage of saline alone (33). Therefore, the results from the present study (**Figure 7.1**), our previous study that investigated effective arginine precursors (3), and the piglet enterocyte work of Wu et al.

(33) taken together provide compelling evidence that citrulline formation limits endogenous arginine synthesis in enterally-fed piglets. The implication of these findings is that the best way to enhance arginine synthesis *in vivo* is to increase citrulline synthesis, particularly at the CPS I step.

Because there was no difference in the rate of proline to ornithine conversion between the two diets (**Figure 7.1**), it is likely that the step in the metabolic pathway between proline and citrulline formation that was most affected by arginine intake was the conversion of ornithine to citrulline. Using a recently described stochastic model (15), citrulline flux is equivalent to the rate of *de novo* citrulline synthesis, because ornithine is not a component of protein and ornithine was not provided in any of the diets, and based on this, proline accounted for 35% and 49% of citrulline synthesis in piglets receiving the +Arg and –Arg diets respectively (**Figure 7.1**). The conversion of ornithine to citrulline measured in the present study, included ornithine formed from all possible precursors, and not just proline; therefore, although there was no effect on this rate of conversion due to arginine intake, the rate of proline-derived ornithine conversion to citrulline may have been affected by diet (**Figure 7.1**). This provides further support for the proposal of a tight channeling of mitochondrially-derived ornithine for citrulline formation (35).

An alternate reason why there was no effect of diet on the rate of ornithine conversion to either citrulline or arginine is that the ornithine isotope may not have equilibrated completely with the intramitochondrial amino acid pool. In humans, it has been suggested that exogenous ornithine is a poor arginine precursor due to a lack of equilibration with the mitrochondrial pool (9); therefore, an intravenously or intragastrically-infused ornithine isotope may not be the most accurate reflection of whole-body ornithine flux. Not only is the formation of ornithine from proline, via ornithine aminotransferase (OAT; EC number 2.6.1.13), a mitochondrial reaction, but the ornithine transcarbamoylase reaction (EC number 2.1.3.3) is also mitochondrial; therefore, if the intragastrically-administered ornithine isotope did not completely equilibrate with the mitochondrial pool, then the isotopic results from the present study may not accurately reflect differences in mitochondrial ornithine formation or metabolism due to diet. Whether exogenously-administered ornithine equilibrates with the intramitochondrial ornithine needs to be experimentally determined, and if it does not, then it may be necessary to develop new experimental approaches to study the mitochondrial metabolism of ornithine, particularly as it relates to arginine synthesis.

Because the P5C reductase (EC number 1.5.1.2) reaction, the final step in proline synthesis, is cytosolic and can occur in the piglet intestine (37), the intragastrically-administered proline isotope was an appropriate choice for studying the whole-body metabolism of both endogenously synthesized and dietary proline. Although citrulline synthesis is mitochondrial, the conversion of citrulline to arginine, which is the only metabolic pathway for citrulline metabolism, is cytosolic; therefore, the isotopic approach used in the present study was also appropriate for studying citrulline metabolism.

7.4.5 *Metabolism of intermediates in the proline to arginine pathway*

Only 27% and 60% of the ornithine formed from proline, for the piglets receiving the +Arg and –Arg diets, respectively, was converted to citrulline and subsequently arginine (**Figure 7.1**). Dillon et al (38) previously described that the conversion from proline to arginine was inefficient in 14 day old piglet enterocytes, with only ~ 7% of the P5C formed from proline, eventually being converted to arginine. Although proline conversion to ornithine was similar for both diets in the present study, 73% and 40% of this ornithine for piglets receiving the +Arg and –Arg diets, respectively (**Figure 7.1**), was metabolized by other pathways: either oxidation to carbon dioxide via the citric acid cycle, conversion back to proline via OAT and P5C reductase, conversion to other amino acids such as glutamate and glutamine via OAT and P5C dehydrogenase (EC number 1.5.1.12), or the synthesis of polyamines by ornithine decarboxylase (EC number 4.1.1.17). The metabolic fates of ornithine have been studied in piglets receiving either an arginine-deficient diet, or the arginine-deficient diet supplemented with ornithine (15), but not in piglets receiving the +Arg diet. Piglets receiving the ornithine-supplemented diet had double the rate of whole-body ornithine flux, a 50% greater rate of ornithine oxidation, a 192% greater rate of ornithine conversion to proline and a numerically greater rate of ornithine conversion to glutamate than piglets receiving the deficient arginine diet (15). In the present study, piglets in the +Arg group had a rate of ornithine conversion to proline that was 327% of the rate in the –Arg group, and the equilibrium of the OAT reaction was clearly shifted towards proline versus ornithine formation in the +Arg group (**Figure 7.1**). Ornithine oxidation was not measured in the present study; however, because ornithine oxidation is related to ornithine flux (15), and the fact that ornithine oxidation in adult humans increased with arginine intake (8,21), it is likely that the piglets in the +Arg group had a greater rate of ornithine oxidation than the piglets in the –Arg group. Together these findings, in combination with the fact that there was a greater extent of metabolism of proline-derived ornithine to metabolites other than citrulline in the +Arg versus –Arg piglets, indicate that when arginine intake is adequate or in excess of the requirements, ornithine is directed away from arginine synthesis and towards the formation of other metabolic products.

7.4.6 *Arginine as a nitric oxide precursor*

Although the rate of [ureido-^{15}N]citrulline formation from [guanido-$^{15}N_2$]arginine has been used in mice (39), growing pigs (22), and humans (19) to measure nitric oxide synthesis, the present study is the first in neonatal animals to use this isotopic technique to study the effect of arginine intake on whole-body nitric oxide synthesis. Piglets in the present study receiving the +Arg diet synthesized twice as much nitric oxide as piglets receiving the –Arg diet (**Figure 7.1**). Previous studies in men (8,9) and mice (39) did not find an effect of arginine intake (8,9) or circulating arginine concentration (39) on nitric oxide synthesis. Other studies, however, reported an effect of arginine intake or status on nitric oxide synthesis. During the acute phases of PPHN, infants had a lower arginine flux and a lower rate of nitric oxide synthesis than when they were recovering from the hypertensive episode (11). Pigs recovering from an experimentally-induced endotoxemia receiving an oral arginine supplement had a much greater rate of nitric oxide synthesis than those receiving an isocaloric alanine supplement (40). Therefore, nitric oxide synthesis appears to be sensitive to arginine intake or status under certain physiological conditions (ie. infancy or pathological states), but not in healthy adults.

In a previous study in enterally-fed piglets, there was a statistical trend for higher plasma nitric oxide concentrations in piglets receiving a generous versus deficient arginine diet (1); however, this could not be confirmed in the present study because there were no differences in plasma nitric oxide concentrations between the two treatment groups (**Table 7.4**). Urinary nitrate excretion was not measured in the present study; however, in the human infants with PPHN, during the recovery period when the rate of nitric oxide synthesis was greater, there was also an increase in urinary nitrate excretion, compared to during the acute PPHN phase (19). Similarly, in rats receiving a diet with either 0, 0.3 of 1% arginine, nitrate excretion increased with increasing arginine intake (41). Therefore, the piglets in the +Arg group, which had a greater rate of nitric oxide synthesis, may also have had a greater rate of nitrate excretion than the –Arg group, resulting in no net effect of arginine intake on plasma nitric oxide concentrations.

Regardless of arginine intake, nitric oxide synthesis accounted for ~13% of whole-body arginine flux in enterally-fed neonatal piglets (**Figure 7.1**). The portion of arginine flux converted to nitric oxide was not affected by arginine intake, despite the fact that both whole-body arginine flux and nitric oxide synthesis were lower in piglets receiving the –Arg versus +Arg diet (**Figure 7.1**), and this provides important evidence that nitric oxide synthesis was driven by arginine availability.

Nitric oxide synthesis accounted for 0.16 – 1.2% of plasma arginine flux in healthy adult humans (8,9,19), and 0.15-0.47% of plasma arginine flux in neonates (19). In mice, the portion of plasma arginine flux accounted for by nitric oxide synthesis ranged from 1.1% to 6.1% (39,42). Therefore, a value of 13% of arginine flux being used for nitric oxide synthesis in the piglets of the present study is substantially higher than most of the values obtained from previous studies. However, in pigs following an experimentally-induced endotoxemia, nitric oxide synthesis represented ~15% of arginine flux and the portion of flux appeared to be independent of arginine supplementation (40). Endotoxemia results in a large increase in nitric oxide synthesis (22,43) and this explains why the pigs in the previous study (40) used such a large portion of their arginine flux for nitric oxide production. However, the piglets in the present study were healthy, and therefore an alternate explanation for the high rate of nitric oxide synthesis is necessary.

An important distinction between the present and previous studies is that in all of previous studies examining nitric oxide synthesis (9,11,19,39,40), the arginine isotope was administered intravenously, whereas in the present study it was delivered intragastrically. Therefore, the present study took into account the arginine that was converted to nitric oxide during first-pass splanchnic metabolism, while previous studies did not (9,11,19,39,40); this shows that the splanchnic region is an important site of nitric oxide synthesis from dietary arginine. Although we did not measure nitric oxide synthesis using an intravenously infused arginine isotope in the present study, in a previous study, using identical diets and methods (2), an intravenously-administered arginine isotope was used and it was found that in piglets receiving the +Arg and –Arg diets respectively, 57% and 8% of the citrulline flux was derived from arginine. Using previously determined fractional net conversions (2) in combination with the citrulline fluxes from the present study (**Figure 7.1**), the rate of arginine to citrulline conversion, using an intravenously-administered arginine isotope, was 63 μmol/(kg·h) (7.7% of arginine flux) in piglets receiving a generous arginine diet and 10 μmol/(kg·h) (2.8% of arginine flux) in piglets receiving the deficient arginine diet. However, the arginine isotope used in the previous study was [4,5-^{3}H]arginine; therefore, the conversion from arginine to citrulline included citrulline derived from arginine metabolized by both the arginase and NOS pathways. The actual rate of arginine to nitric oxide conversion would be less than the measured rate of arginine to citrulline conversion measured using [4,5-^{3}H]arginine, because for both diets there was a considerable rate of arginine metabolism by arginase pathway [intravenous arginine to ornithine conversion rate of 556 μmol/(kg·h) for the +Arg diet and 141 μmol/(kg·h) for the –Arg diet] (2). This means that if the arginine isotope in the present study had been administered intravenously, the portion of arginine flux accounted for by nitric oxide synthesis in the enterally-fed piglets of the present study would have been similar to previous estimates in mice and humans (9,11,19,39,40). However, in order to verify that first pass intestinal or splanchnic metabolism made a major contribution to whole-body nitric oxide synthesis, additional research using intraportal or intravenous arginine infusions, to isolate the effects of first-pass intestinal and splanchnic metabolism respectively, are required.

These data with regards to the extensive metabolism of dietary arginine to nitric oxide during first-pass splanchnic metabolism data complement previous findings by us and others. In adult men, when a [guanido-$^{15}N_2$]arginine isotope was given either intravenously or intragastrically, the amount of $^{15}NO_3$ excreted in the urine was ~4 times greater when the isotope was given intragastrically (44), indicating that ~75% of arginine conversion to nitric oxide may occur during first-pass splanchnic metabolism. The activity of NOS has been detected in the intestine of piglets of all ages (45,46), and both the portal-drained viscera and liver of ~25 kg fasted pigs (22) and of pigs recovering from endotoxemia (40) were sites of nitric oxide production. We have previously shown that ~50% of dietary arginine was extracted during first-pass splanchnic metabolism, with the majority being extracted during first-pass intestinal metabolism (3,7). Based on low first-pass splanchnic conversion rates of arginine to urea (3), and on very low intestinal arginase activities in suckling piglets (45), urea production did not account for a substantial portion of the intestinally-extracted arginine, particularly in piglets receiving a deficient arginine diet (3). However, from the present study, the production of nitric oxide synthesis could account for a substantial portion of the intestinally-extracted arginine, and one use for this intestinally-produced nitric oxide may be the regulation of intestinal blood flow (46).

The present study found that piglets receiving a deficient arginine diet had lower rates of nitric oxide synthesis than piglets receiving a generous arginine diet, and it is likely that a large portion of this nitric oxide synthesis was intestinal. This could be one mechanism for the protective effect of arginine supplementation in premature human neonates against the development of NEC (12). However, these results also suggest that arginine supplements should be administered orally or intragastrically in order to have the greatest possible effect on nitric oxide synthesis.

7.4.7 *Conclusion*

The major objectives of the present study were to study: 1) total arginine synthesis, 2) the contribution of proline to whole-body arginine synthesis, 3) the limiting steps in arginine formation from proline, and 4) to determine whether nitric oxide synthesis was affected by arginine intake. Regardless of arginine intake, proline conversion to arginine accounted for ~60% of endogenous arginine synthesis and it was citrulline formation that was limiting to arginine synthesis from proline. Although piglets receiving the –Arg diet had a rate of endogenous arginine synthesis 79% greater than in piglets receiving the +Arg diet [120 versus 67 μmol/(kg·h)], their rate of nitric oxide synthesis was only 44% of the rate in piglets receiving the +Arg diet. The unique multi-isotope methodology employed in the present study allowed us to simultaneously study many aspects of arginine metabolism in neonatal piglets, and will be a useful experimental tool for future studies in both piglets and human neonates.

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8.0 SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1 Sites of endogenous arginine synthesis in enterally and parenterally-fed piglets

One of the primary objectives of this thesis was to examine sites of endogenous arginine synthesis in neonatal piglets. In the first study [Chapter 3.0, (1)], the contribution of first-pass hepatic metabolism to whole-body arginine synthesis was investigated in enterally-fed piglets receiving either a deficient [0.20 g/(kg·d)] or generous [1.80 g/(kg·d)] intake of arginine. Regardless of arginine intake, first-pass hepatic metabolism did not contribute to whole-body arginine synthesis (1). This is in contrast to first-pass intestinal metabolism which accounted for 42 and 63% of whole-body arginine synthesis, in piglets receiving the deficient and generous intakes of arginine respectively (2). First-pass hepatic metabolism was studied [Chapter 3.0, (1)]; however, because portal and arterial blood mix in the liver sinusoids (3), and therefore the nutrients entering the liver by both vessels are metabolized in a similar manner. Based upon rat data (4-7) it is likely that hepatic metabolism in general was not involved in arginine synthesis. The results from this study in neonatal piglets produced similar results to mass-balance and ligation studies in adult and growing rodents (4-7), which showed that the rodent liver did not release arginine, despite containing all of the urea cycle enzymes (8). Therefore, the metabolic importance of the urea cycle enzymes in the liver is not arginine synthesis, but rather ammonia detoxification, an observation that is further supported by the observation that intermediates in the urea cycle are tightly channeled from one enzyme to the next (9).

Because the liver was not a site of arginine synthesis this explains why individuals with inborn errors in urea cyc[illegible]metabolism that received a liver transplant continued to have a dietary requirement for arginine (10,11). Although the previously absent enzyme from the hepatic urea cycle was now present in the transplanted liver, a functional form of that enzyme remained absent from other tissues such as the intestine and therefore endogenous arginine synthesis could not occur. The metabolic requirement for arginine has not been experimentally determined in humans of any age, and therefore the amount of dietary arginine that needs to be provided to humans with inborn errors involving arginine synthesis and metabolism is unknown and must be experimentally-determined.

First-pass intestinal metabolism only accounted for 42 – 63% of whole-body arginine synthesis from proline (2); therefore, the tissue sites responsible for the remaining 37 – 58% of arginine synthesis still required elucidation, but was due to the metabolism of circulating arterial proline [Chapter 3.0, (1)]. The strong linear relation between mucosal mass and arginine synthesis from circulating proline, in parenterally-fed piglets [Chapter 4.0, (12)], strongly suggested that the intestinal metabolism of circulating arterial precursors was responsible for at least a portion of the endogenous arginine synthesis in enterally-fed piglets that occurred during peripheral metabolism.

Other tissues such as the muscle or the kidney may also be involved in whole-body arginine synthesis in neonatal piglets. Although there is detectable activity of both argininosuccinate synthetase (EC number 6.3.4.5) and argininosuccinate lyase (EC number 4.3.2.1) (13), the activities of several of the other enzymes in the arginine synthetic pathway from proline, specifically proline oxidase (EC number 1.5.99.8), ornithine aminotransferase (OAT; EC number 2.6.1.13) and ornithine transcarbamoylase (OTC; EC number 2.1.3.3) are only expressed in certain tissues. Neither the kidney nor the muscle has the enzymatic capacity for the entire proline to arginine conversion in enterally-fed piglets, with the kidney lacking OTC activity (14) and the muscle lacking proline oxidase (15). Compared to the intestine, both tissues have extremely low OAT activities (16). Therefore, it is likely that any contribution that the muscle or kidney would make to whole-body arginine synthesis would be limited to after ornithine or citrulline formation, respectively, and the potential contribution of these tissues to whole-body arginine synthesis would not have been detectable using the intravenously infused proline isotope [Chapter 3.0, (1)]. In order to confirm that it was the intestinal metabolism, and not either renal or muscle metabolism, of arterial proline that was responsible for the unaccounted for portion of whole-body arginine synthesis from proline (2), additional research is necessary. Mass-balance experiments, similar to those done in ~25 kg pigs (17) and mice (18) to measure nitric oxide synthesis by individual tissues (liver, portal-drained viscera, kidney and hindquarter muscle), could be conducted in neonatal piglets during a proline isotope infusion to measure each individual organ's contribution in the proline to arginine metabolic pathway.

There is a large potential for future research using the multi-isotope infusion procedures used in the final study (Chapater 7.0), that would greatly advance our knowledge of the role of inter-organ metabolism in neonatal arginine synthesis. By combining mass-balance procedures to isolate liver, portal-drained viscera, renal and muscle metabolism (17,18) with the multi-isotope infusion procedures (Chapter 7.0), it will be possible to isolate and quantify the role that each tissue plays in proline to arginine conversion. Therefore, although the muscle and kidney are unlikely to be capable of the entire proline to arginine conversion, as previously discussed, if they are involved in even a portion of the synthetic pathway, their role could be elucidated using this proposed methodology. The isotopic infusion technique used to isolate the contribution of first-pass intestinal (2) and hepatic metabolism [Chapter 3.0, (1)] allowed us to conclude that first-pass intestinal metabolism was required for 42 – 63% of whole-body arginine synthesis and that the entire conversion of proline to arginine could not occur during first-pass hepatic metabolism; however, using this method we could not determine: a) whether the entire conversion of proline to arginine occurred during first-pass intestinal metabolism, or b) whether first-pass hepatic metabolism was involved in a portion of the arginine synthetic pathway following the initial conversion of proline to P5C. In order to answer these questions, the multiple stable isotope infusion methodology from Chapter 7.0 could be repeated using intragastric and intraportal infusions, to determine which conversions occur during first-pass intestinal metabolism (2), and intraportal and intravenous infusions, to determine whether first-pass hepatic metabolism is involved in the synthesis and subsequent release of any of the intermediates of whole-body arginine synthesis [Chapter 3.0, (1)].

We hypothesized that the intestinal metabolism of circulating precursors would be an important source of endogenous arginine synthesis. The second study [Chapter 4.0, (12)] used the unique approach of combining parenteral feeding with a continuous infusion of either saline or glucagon-like peptide 2 (GLP-2) as an experimental tool to study the effect of differing intestinal morphology on the use of circulating precursors for arginine synthesis. Parenteral feeding alone resulted in intestinal atrophy (19) and a decrease in intestinal blood flow (20) which could be reversed by the administration of GLP-2 (21,22). The GLP-2 receptor is expressed primarily in the proximal small intestine (23), and therefore the trophic effects of GLP-2, including the increase in intestinal blood flow (24), and increased rates of protein synthesis (25) appeared to be localized to the small intestine as well. By having one group of piglets (GLP-2 infused) with 'healthier' small intestinal morphology and higher intestinal blood flow than the second group of piglets (saline infused), the difference in rates of arginine synthesis between the two groups were proposed to be attributable to differences in small intestinal metabolism of circulating precursors. This hypothesis was supported by the fact that the rate of arginine synthesis from proline in parenterally-fed piglets was strongly related to both the mucosal mass ($R^2 = 0.72$) and villus height ($R^2 = 0.65$) [Chapter 4.0, (12)]. Moreover, the rate of endogenous arginine synthesis was 100% greater in piglets receiving the GLP-2 versus saline infusion [Chapter 4.0, (12)]. Compared to the rate of arginine synthesis from circulating proline determined in Chapter 3.0 (1), the parenterally-fed piglets, even those that received a continuous infusion of GLP-2, had rates of arginine synthesis that were 75 – 84% lower than in enterally-fed piglets receiving an arginine-deficient diet [Chapter 4.0, (12)]. Therefore, despite improvements in gut morphology and an increase in the rate of arginine synthesis from circulating proline due to GLP-2 infusion, there was still clearly a limitation in the rate of endogenous arginine synthesis from circulating proline in parenterally-fed piglets compared to enterally-fed piglets.

If the reason for the difference in the rates of arginine synthesis from circulating proline between parenterally and enterally-fed piglets was strictly due to the reduction in intestinal blood flow (20) and intestinal atrophy (19,22), then the piglets receiving the GLP-2 infusion should have been capable of similar rates of endogenous arginine synthesis from circulating proline as enterally-fed piglets. This assumption is based on the fact that although piglets receiving parenteral nutrition alone had lower rates of intestinal blood flow [3.13 L/(kg·h)] (21) and lower jejunum mucosal masses [19 mg/(kg·cm)] [Chapter 4.0, (12)] than either the parenterally-fed piglets receiving GLP-2 or enterally-fed piglets, piglets receiving parenteral nutrition + GLP-2 had jejunum mucosal weights that were similar to enterally-fed piglets of the same age [enterally-fed: 33 mg/(kg·cm); parenterally-fed + GLP-2: 29 mg/(kg·cm)] [Chapter 4.0, (12,19)] and also had similar rates of portal vein blood flow [enterally-fed: 3.57 L/(kg·h); parenterally-fed + GLP-2: 3.81 L/(kg·h)] (21,26). However, because improving intestinal blood flow and mucosal weights did not achieve the hypothesized effect on rates of endogenous arginine synthesis [Chapter 4.0, (12)], there must be other factors that limit endogenous arginine synthesis in parenterally-fed piglets.

The parenteral nutrition may have had an adverse effect on other organs, including the liver, kidney or muscle, and if any of these organs affected by parenteral nutrition were involved in arginine synthesis from circulating precursors, then this would provide an explanation for the low rate of arginine synthesis from circulating proline in parenterally versus enterally-fed piglets. In addition to having adverse effects on intestinal morphology (19,22), parenteral nutrition has also been shown to result in apoptotic liver injury (27) and cholestasis (28,29) in neonatal piglets; however, the liver is not a site of endogenous arginine synthesis from proline [Chapter 3.0, (1)], and thus the parenteral nutrition-induced liver damage should not have affected rates of arginine synthesis from proline. Neither the kidney nor the muscle has the enzymatic capacity to convert proline to citrulline; therefore, even if there was an effect of parenteral feeding on renal or muscle amino acid metabolism, it would not explain the greater rate of arterial proline conversion to arginine in enterally versus parenterally-fed piglets.

It is hypothesized that the reason that there was such a substantial difference in the rate of arterial proline conversion to arginine between enterally [Chapter 3.0, (1)] and parenterally-fed [Chapter 4.0, (12)] piglets is that the parenteral nutrition resulted in changes in the activity of one or more of the enzymes involved in arginine synthesis that could not be improved by GLP-2 administration. Alternatively, the rate of endogenous arginine synthesis in enterally-fed piglets was related to the intestinal mitochondrial N-acetylglutamate (NAG) concentrations (30), and it may be that parenterally-fed piglets have lower NAG concentrations, which limits arginine synthesis. Neither the intestinal activities of the arginine synthetic enzymes nor the intestinal NAG concentrations have been measured in parenterally-fed piglets. In order to advance our understanding of arginine metabolism in parenterally-fed piglets, this is clearly an area where additional research is required.

Supplementing an arginine-deficient diet with citrulline in parenterally-fed piglets, and using a citrulline isotope to measure arginine synthesis, may provide interesting results with regards to the potential role of renal metabolism for neonatal arginine synthesis. The site of citrulline to arginine conversion portion of endogenous arginine synthesis is primarily renal in weaned mammals (6,31), and the enzymatic capacity for renal arginine synthesis from citrulline does exist in young piglets (32); however, the role of the kidney in arginine synthesis has not been directly studied in neonates. Based on the extremely low rates of proline to arginine conversion in parenterally-fed piglets, which was shown to be largely dependent on mucosal mass [Chapter 4.0, (12)], it may be assumed that the parenterally-fed intestine did not release large amounts of citrulline to be converted to arginine by the kidney and only synthesized small amounts of arginine from circulating or intestinally-synthesized citrulline. Therefore, if renal metabolism is capable of metabolically important rates of citrulline conversion to arginine in parenterally-fed piglets, it would be expected that citrulline addition to a parenteral formula with low levels of arginine would result in an improvement in whole-body arginine status, because these effects were observed in enterally-fed piglets [Chapter 5.0, (33)]. Although the intestinal consequences of parenteral nutrition have been well-described in neonatal piglets (19,22), to the best of my knowledge, there has been little or no research investigating the effects of parenteral nutrition on renal function in neonates, although renal amino acid concentrations are different in parenterally-fed versus enterally-fed neonates (34).

8.2 Limitations in endogenous arginine synthesis in neonates

The second major objective of this thesis was to determine which portion(s) of the arginine synthetic pathway was limiting to endogenous arginine synthesis in neonatal piglets. Although whole-body arginine synthesis from proline was greater in piglets receiving a deficient arginine diet than in those receiving a generous arginine diet (2), there were still clear differences in the indicators of whole-body arginine status between the two groups. With regards to human health, an inability to synthesize enough arginine could pre-dispose infants to conditions such as necrotizing enterocolitis (35,36) or persistent pulmonary hypertension of the neonate (37). Furthermore, in a study where arginine was added to a piglet milk-replacer, piglets receiving the arginine supplemented diet had a greater rate of growth than those receiving the control diet (38). The limitations in the capacity of endogenous arginine synthesis could have important implications for both humans and piglets and therefore the identification of these limitations will allow dietary strategies to be devised to optimize arginine nutrition.

The arginine-deficient diet was supplemented with equimolar amounts of proline, ornithine, citrulline and arginine to determine which of the arginine precursors could be effectively converted to arginine [Chapter 5.0, (33)]. Because citrulline was equally as effective as arginine at decreasing plasma ammonia and urea concentrations and increasing plasma arginine concentration and whole-body arginine flux, the limitation in the metabolic pathway between proline and arginine in enterally-fed piglets was concluded to be citrulline formation [Chapter 5.0, (33)]. Furthermore, the rate of proline conversion to citrulline was the same as the rate of proline conversion to arginine, regardless of arginine intake (Chapter 7.0), providing clear isotopic support and quantification of the metabolic changes described in Chapter 5.0 (33). These findings supported work by Wu et al. (30), which found that the low NAG concentrations in suckling piglet enterocytes were a limiting factor for both citrulline and arginine formation, and that if an NAG analog, N-carbamoylglutamate (NCG) was provided to suckling piglets, then the intestinal rate of arginine and citrulline formation increased, plasma arginine concentrations increased, and piglet weight gain was greater than in piglets receiving a saline control. It would be of interest to determine whether NCG addition to the arginine-deficient diet, would be as effective as citrulline addition

[Chapter 5.0, (33)] in promoting whole-body arginine synthesis, and whether there was a difference in precursor effectiveness between enterally and parenterally-fed piglets.

Neither proline nor ornithine addition to the arginine-deficient diet improved arginine status in comparison to the arginine-deficient diet alone. In Chapter 6.0 (39), the hypothesis that the reason that ornithine was not an effective precursor because it was extensively converted to other metabolites such as carbon dioxide and proline was tested. The simultaneous administration of α-ketoglutarate and ornithine in a 1:2 ratio was more effective than ornithine administration alone in promoting arginine synthesis in adult humans (40); this was also examined. Regardless of whether α-ketoglutarate was added to the diet, the rate of arginine synthesis from ornithine was the same and additionally, there were no difference in whole-body arginine status between any of the treatment groups [Chapter 6.0, (39)], confirming the findings from Chapter 5.0 (33). Compared to piglets receiving a diet without ornithine, the supplemental ornithine was extensively oxidized to carbon dioxide during peripheral metabolism, and during first-pass intestinal metabolism there was a significant amount of proline synthesis from the ornithine. The inability of ornithine to extensively replace dietary arginine may also be partially explained by the observation that exogenously administered ornithine does not appear to equilibrate completely with the mitochondrial ornithine pool [Chapter 7.0,(41,42)]. Because ornithine formation from both proline and glutamine/glutamate and ornithine metabolism to citrulline occur in mitochondrial reactions, if exogenously administered ornithine does not effectively enter the mitochondria then it cannot be an effective precursor for arginine synthesis.

The activities of the enzymes involved in arginine synthesis and metabolism have been extensively studied in the enterocytes of suckling and recently weaned piglets (32,43-45). However, to date there has been no research examining the effects of deficient versus generous arginine intake on the activities of these enzymes, and this research may provide a better understanding of which enzymes are involved in the increased rate of arginine synthesis in response to dietary arginine deficiency [Chapter 7.0, (2)] and needs to be conducted. Although the rate of proline conversion to citrulline was greater in piglets receiving the deficient arginine diet in Chapter 7.0, the rates of proline to ornithine and ornithine to citrulline conversion were not affected by arginine intake and therefore the metabolic step that was regulated by arginine intake could not be determined, although we hypothesized that the conversion of proline-derived ornithine to citrulline was greater in piglets receiving the deficient versus generous arginine diet (Chapter 7.0). Enzyme data may be useful in clarifying which steps in the metabolic pathway are responsive to arginine intake.

8.3 **Other aspects of arginine metabolism related to this thesis**

8.3.1 *Total arginine synthesis in enterally and parenterally-fed piglets*

In the earlier experiments in this thesis [Chapter 3.0 and 5.0, (1,33)], the rate of proline conversion to arginine in enterally-fed piglets was used as a measure of whole-body arginine synthesis, based on previous research that found no measurable conversion of enterally-administered glutamate to arginine (2). However, in the final study (Chapter 7.0), we showed that only 60% of arginine synthesis, regardless of arginine intake, could be accounted for by arginine synthesis from proline; therefore, the rates of whole-body arginine synthesis from the previous studies were probably underestimated. However, because in enterally-fed piglets the portion of total arginine synthesis accounted for by proline conversion was constant regardless of arginine intake, the comparisons made between treatment groups for the rates of arginine synthesis were still valid. By dividing the rates of proline to arginine conversion by 0.60, the total rate of arginine synthesis from studies 3.0 and 5.0 can be calculated. Using the proline to arginine conversion rates from Chapter 3.0, the rate of arginine synthesis from circulating precursors is estimated to be 79 μmol/(kg·h) and 26 μmol/(kg·h), in the –Arg and +Arg piglets respectively, which is equivalent to 66 and 39%, respectively, of whole-body arginine synthesis (Chapter 7.0). Likewise, when the rates of total arginine synthesis are calculated for the piglets in Chapter 5.0 (33) receiving either the basal (equivalent to –Arg) or +Arg diets, the values of 125 and 67 μmol/(kg·h) are virtually identical to the values obtained in the subsequent study (Chapter 7.0).

In parenterally-fed piglets, the contribution of proline to whole-body arginine synthesis still requires experimental determination, and this can be achieved by using a citrulline isotope to measure the total rate of arginine synthesis as was done in enterally-fed piglets (Chapter 7.0). Assuming that the portion of total arginine synthesis accounted for by proline to arginine conversion is similar in enterally and parenterally-fed piglets, piglets in the +Sal and +GLP-2 groups respectively had rates of total arginine synthesis of only 11 and 20 μmol/(kg·h) [Chapter 4.0, (12)].

8.3.2 *The need to experimentally determine the arginine requirement*

Arginine has been previously identified as an indispensable amino acid in both enterally (46,47) and parenterally-fed (46) piglets. Even the combination of the maximum rate of proline conversion to arginine [0.30 g/(kg·d)] (Chapter 7.0) and arginine intake from sow's milk [0.42 g/(kg·d)] (48) is calculated to only meet 60% of the metabolic arginine requirement; therefore, as discussed in Chapter 7.0, there must be other arginine precursors in neonatal piglets, because milk-fed piglets do grow and do not become hyperammonemic. The maximum rate of citrulline conversion to arginine, which represents the rate of arginine synthesis from all arginine precursors, is equivalent to ~0.50 g/(kg·d), which when combined with arginine intake from sow's milk still only provides ~75-85% of the metabolic arginine requirement (48,49). These findings may provide a metabolic basis for the reason why arginine supplementation to a milk-replacer diet was effective at improving piglet growth (38). In light of these findings in piglets, it is necessary to evaluate the metabolic arginine requirement and rates of endogenous arginine synthesis in human neonates, particularly pre-mature neonates, to determine whether the combination of intake, be it from formula or milk, and endogenous synthesis are enough to meet the metabolic arginine requirement and support optimal health.

The extremely low rate of endogenous arginine synthesis from proline in parenterally-fed piglets [Chapter 4.0, (12)] means that arginine is an indispensable amino acid in parenterally-fed piglets and that the parenteral solution must provide the majority (~95%) of metabolic arginine requirement. The arginine content of parenteral solutions is variable (47 – 122 mg/g total amino acid) (**Table 1.1**) (50), and if these parenteral solutions were administered at a rate sufficient to provide 15 g amino acids/(kg·d) to piglets, some of these solutions would not provide enough arginine to meet the estimated metabolic arginine requirement of 1.2 g/(kg·d) (48,49). Assuming the arginine requirement relative to protein requirement of piglets is similar to in humans, as suggested by the similar composition of pig and human tissue (**Table 1.1**) (51), some parenteral solutions given to human neonates may contain sub-optimal levels of arginine and therefore it is important that the arginine requirement of neonates be quantified.

Unlike many of the other indispensable amino acids, including lysine (52), threonine (53), methionine (54) and the branched chain amino acids (55), where the dietary parenteral requirement is less than the enteral requirement, the dietary parenteral requirement of arginine appears to be greater than the enteral requirement. This is evidenced by the fact that in the study where parenteral feeding was used [Chapter 4.0, (12)], the lowest arginine content that could be safely administered was 0.60 g/(kg·d) (49), and the piglets receiving this diet had very similar indices of whole-body arginine status to piglets receiving the arginine-deficient enteral diet containing 0.20 g/(kg·d) arginine [Chapters 3.0, 5.0, 6.0, 7.0; (1,2,33,39)]. Unlike the other indispensable amino acids that have been studied in neonatal piglets, the arginine requirement has not been determined using the indicator amino acid oxidation technique (56) in parenterally-fed piglets. Ball et al (47) used the indicator oxidation to determine the arginine requirement in milk-replacer-fed piglets and determined that the arginine requirement was 22 mg/g protein; however, neither the feed intake nor daily protein intake of these piglets was provided in the manuscript (47). Based on a total amino acid intake of 15 g/(kg·d), the daily rate of amino acid intake in all studies in this thesis, this requirement is only equivalent to 0.33 g/(kg·d), which is lower than even the estimated intake of arginine sow's milk [0.42 g/(kg·d)] (48). Because the level of arginine provided by sow's milk is believed to be limiting to piglet growth (38), this determined requirement is likely to be an underestimation of the true dietary arginine requirement (47).

In a preliminary, unpublished study by our group using the indicator amino acid oxidation method to measure the arginine requirement in parenterally-fed piglets, there was no relationship between the level of arginine intake and the rate of phenylalanine oxidation; and therefore arginine requirements could not be determined in this manner. The indicator amino acid oxidation technique may not be the optimal method to determine the dietary arginine requirement. In piglets, arginine is somewhat unique as an indispensable amino acid in that there is some capacity for its endogenous synthesis, which is inversely related to its intake (2); therefore, there is not a simple relation between arginine intake and rates of protein synthesis, which subsequently determines the rate of phenylalanine oxidation. Arginine also has many other metabolic fates in addition to protein synthesis (48), and therefore the partitioning of arginine between these different metabolic fates will have an effect on the responsiveness to indicator amino acid oxidation to arginine intake.

When determining rates of amino acid oxidation, a bicarbonate retention factor must be applied to correct for the amount of carbon dioxide produced by amino acid oxidation that is not exhaled, but remains as bicarbonate. The bicarbonate retention factor for piglets has been experimentally determined in parenterally-fed piglets receiving a commercial parenteral solution (57), and has been successfully applied in the determination of the other indispensable amino acid requirements (52-55,58,59). However, because the carbamoyl phosphate synthetase (EC number 6.3.4.16) reaction of the urea cycle and arginine synthesis involves the condensation of bicarbonate with ammonia, it is possible that the bicarbonate retention factor may have been affected by arginine intake, which would have affected the calculation of the rates of arginine oxidation. It would be of interest to determine the effect of arginine intake on the bicarbonate retention factor to see if this allowed for a better outcome in using the indicator amino acid oxidation method to measure the arginine requirement. Because of the difficulties in measuring the arginine requirement using the indicator amino acid oxidation method, factorial approaches have been used to estimate the metabolic arginine requirement in parenterally (49) and enterally-fed (48) piglets.

Finally, with the exception of Chapter 4.0 (12), where parenterally-fed piglets and a single low arginine diet were used, all experiments in enterally-fed piglets examined arginine metabolism at one of two arginine intakes: a generous intake estimated to provide 150% of the metabolic arginine requirement (48,49), and a deficient intake estimated to provide ~17% of the metabolic arginine requirements (48,49). Differences in arginine synthesis rates and conversion rates to other metabolites [Chapters 3.0, 5.0, 7.0, (1,33)] were evident between the two levels of arginine intake; however, it is necessary to study arginine synthesis and metabolism over a range of arginine intakes to determine the whether the response to arginine intake for each of the parameters is linear or quadratic and the arginine intake level at which the rate of arginine synthesis reaches the basal and maximal levels. Studying arginine metabolism over a wide range of intakes will also help to elucidate optimal levels of dietary arginine provision during both parenteral and enteral feeding.

8.3.3 *Relationship between proline and arginine metabolism and possible sparing effects*

An important consideration when determining the arginine requirement is that a portion of the arginine requirement can be met by its precursor amino acids. Proline, as the major arginine precursor [Chapter 7.0, (2)], spared a portion of the arginine requirement, but only up to a maximum of ~1.8 mmol/(kg·d) (Chapter 7.0). The metabolic arginine requirement for week-old piglets has been estimated at ~1.2 g/(kg·d) or 6.9 mmol/(kg·d) (48); therefore proline can spare the metabolic arginine requirement by ~25%. Relative to the estimated daily metabolic arginine use, sow's milk and human milk both provide relatively low amounts of arginine and only trace amounts of both ornithine and citrulline, but contain large amounts of proline (48,60,61); therefore, this determination of the ability of proline to spare a portion of the metabolic arginine requirement is of nutritional significance.

In enterally-fed piglets receiving 0.90 g/(kg·d) arginine, an intravenously administered arginine isotope was converted to proline, and the rate of this conversion was greater in piglets receiving 0.07 g/(kg·d) proline than in those receiving 0.95 g/(kg·d) proline (62). In a second study in enterally-fed piglets [receiving 0.90 g/(kg·d) arginine], the whole-body rate of proline to arginine conversion was similar to the whole-body rate of arginine to proline conversion (63). From these studies, it is evident that arginine can be converted to proline, and thus it is possible that arginine can spare a portion of the metabolic proline requirement. In Chapter 5.0 (33), we showed that when either arginine or citrulline was added to the arginine deficient diet, resulting in improved arginine status, there was a decrease in the amount of proline converted to arginine. These results suggest that the ability of arginine to spare a portion of the metabolic proline requirement is dependent on arginine intake and status: if arginine status was adequate then there was only a basal level of proline conversion to arginine, whereas when arginine status was less favorable (deficient arginine diet), more proline was diverted to arginine. In order to quantify the amount of the metabolic proline requirement that can be spared by arginine, two key pieces of information are necessary: the metabolic proline requirement and the maximum rate of arginine to proline conversion.

In enterally-fed piglets, the dietary proline requirement was estimated using the indicator amino acid oxidation method to be 55 – 70 mg/g protein (47). Assuming 15 g/(kg·d) of total amino acid intake, the intake used in all of the studies of this thesis, the dietary proline requirement in enterally-fed piglets would be equivalent to 0.83 – 1.05 g/(kg·d). A factorial estimate of the metabolic proline requirement has not been previously conducted; however, it may be at least partially extrapolated. The proline composition of piglet tissue is 60 mg/g protein (**Table 1.1**) (51), and therefore assuming a rate of protein accretion of 27.2 g/d (64), in a 2.5 kg piglet, the proline requirement for protein synthesis would be 1.63 g/d or 0.66 g/(kg·d). The use of proline for arginine synthesis ranged from 42 – 74 μmol/(kg·h) [0.12 – 0.20 g/(kg·d)] in piglets receiving a proline-rich diet [1.25 g/(kg·d)]. The effect of proline intake on this rate of conversion has not been experimentally determined and therefore may be less in cases of proline deficiency. Other metabolic uses for proline, including the conversion to other amino acids such as glutamate and glutamine and obligatory oxidation still require experimental determination. Therefore, the metabolic requirement for proline is greater than 0.78 g/(kg·d), which is similar to the estimate of the dietary requirement using the indicator amino acid oxidation method (47).

Although the rate of arginine to proline conversion has been previously described (62,63), in both of the previous studies there were major flaws with the calculation of the rate of arginine conversion to proline, that brings into question the validity of previous estimates. In the first study (62), the conversion of [U-^{14}C]arginine to proline was measured; however when calculating the molar conversion rate, the fractional net conversion rate of arginine to proline was multiplied by the precursor (arginine) flux and not by the product (proline) flux, which is the established method for calculating molar conversion rates (65,66). Also, in this study (62), the isotope was infused intravenously and therefore would underestimate the whole-body rate of conversion if first-pass intestinal metabolism is important for this conversion. In the second study, the rate of arginine to proline conversion was not directly measured, with an arginine isotope where the label was associated with the 5-carbon backbone of amino-nitrogen group isotope, but was extrapolated by multiplying the rate of arginine to ornithine conversion by the portion of ornithine flux that was converted to proline (63). However, the rate of arginine to ornithine conversion was not directly measured either, but the fractional net conversion of arginine to urea was assumed to be equivalent to the fractional net conversion of arginine to ornithine because when urea was formed from arginine, an equimolar amount of ornithine was also produced (63). Although the assumption that ornithine and urea are formed in equimolar proportions from arginine is valid, the fractional net conversion rate represents the portion of the product amino acid flux arising from the precursor amino acid; therefore, unless the urea flux was equivalent to the ornithine flux, then the fractional net conversion of arginine to urea was not equivalent to the fractional net conversion of arginine to ornithine. Urea flux has not been reported in piglets; however, in humans, urea flux is 3.5 – 9 times greater than ornithine flux (67); and therefore the previously calculated arginine to proline conversion rate in piglets (63) is unlikely to be correct.

Using the data presented from this thesis, the rate of arginine to proline conversion can be partially calculated. If the fractional rate of arginine to ornithine conversion determined using the intravenous [4,5-^{3}H]arginine infusion in Chapter 3.0 (1) (+Arg = 128% of ornithine flux; -Arg = 74% of ornithine flux), is multiplied by the ornithine flux determined in Chapter 7.0 [+Arg = 452 μmol/(kg·h); -Arg = 191 μmol/(kg·h)], then the molar rate of arginine to ornithine conversion is 579 and 141 μmol/(kg·h) in piglets receiving the +Arg and –Arg diets respectively can be calculated. Using the same approach as described by Bertolo et al (63), and outlined earlier, to calculate the rate of arginine to proline conversion with the proline to ornithine conversion data and ornithine flux data from Chapter 7.0, this rate was equivalent to 251 and 44 μmol/(kg·h) [0.69 and 0.12 g/(kg·d)] in the +Arg and –Arg piglets. An important factor to note, however, is that because the conversion of arginine to ornithine was measured using an intravenous arginine isotope [Chapter 3.0, (1)], it did not take into account the role of first-pass splanchnic metabolism on this conversion and is therefore likely to be a slight underestimate. The maximal rate of the arginine to proline conversion is also likely greater than 0.69 g/(kg·d), because these piglets received a generous intake of proline [1.25 g/(kg·d)] and it has been shown that the rate of conversion is greater when proline intake is low (62). Therefore, it appears that arginine can spare a large portion of the proline requirement when arginine is provided above its metabolic requirement.

Similar to the net rate of arginine synthesis from citrulline (Chapter 7.0), the direction of the flow of substrates through the proline to arginine synthetic pathway is dependent on arginine intake. Using the arginine to proline conversion rates calculated above in combination with the proline to arginine conversion rates [+Arg = 42 μmol/(kg·h); -Arg = 74 μmol/(kg·h)](Chapter 7.0), there was a net formation of proline from arginine [209 μmol/(kg·h)] in the +Arg piglets and a net formation of arginine from proline [30 μmol/(kg·h)] in the –Arg piglets. The net formation of arginine from proline is ~75% of the rate of net arginine synthesis from citrulline in the –Arg piglets (Chapter 7.0), once again confirming that proline is the primary arginine precursor (Chapter 7.0). Because of the inter-relationships between arginine and proline metabolism and the apparent ability to spare each others' requirement, which is dependent on the intake of each of the two amino acids, it may be necessary to provide an arginine + proline requirement instead of individual arginine and proline requirements, as is done for the methionine + cysteine and phenylalanine + tyrosine requirements.

8.3.4 *Arginine conversion to other metabolites and the role of first-pass splanchnic metabolism*

Another area with regards to neonatal arginine metabolism that still requires elucidation is the effect of arginine intake on arginine metabolism to ornithine via arginase (EC number 3.5.3.1), and the subsequent metabolism of the arginine-formed ornithine either to arginine or to carbon dioxide. Although the conversion of circulating arginine to ornithine and citrulline were measured in one study (Chapter 3.0), additional research is necessary to isolate the involvement of the different tissues in arginine metabolism. In humans, oxidative arginine catabolism, as measured by the rate of ornithine oxidation, increased with increasing arginine intake (67,68), and was the major way in which arginine metabolism was regulated in response to arginine intake. From Chapter 3.0 (1), the fractional conversion of arginine to ornithine was greater in the piglets receiving the generous arginine diet, suggesting that arginine catabolism is also responsive to arginine intake in enterally-fed piglets. Furthermore, in piglets, ornithine oxidation appeared to be related to ornithine flux (Chapter 6.0) (39); therefore, although ornithine oxidation was not measured in piglets receiving the generous arginine diet, it would be expected to be greater than piglets receiving the deficient arginine diet based on the differences in the ornithine fluxes between the two diets (Chapter 7.0).

First-pass splanchnic metabolism extracted ~52% of dietary arginine, regardless of arginine intake [Chapter 5.0, (33)], and the majority of this extraction was likely intestinal (63). Arginine conversion to urea does not appear to be a significant metabolic fate of this extracted arginine based on enzymatic data (44) and similar rates of arginine to urea conversion, regardless of whether the arginine isotope was administered intravenously or intragastrically [Chapter 5.0, (33)]. The portion of arginine flux converted to nitric oxide (~13%) when the arginine isotope was administered intragastrically (Chapter 7.0) was much greater than the estimates of human adults and neonates (> 2%) where the isotope was administered intravenously (42,67,69,70), suggesting that a portion of the extracted dietary arginine was used for nitric oxide synthesis. However, the amount of nitric oxide synthesis occurring during first-pass intestinal or splanchnic metabolism was not directly measured. Further research is warranted to determine how much of the splanchnic-extracted arginine is used for each of the possible metabolic fates of arginine, such as protein, nitric oxide and polyamine synthesis.

A [1-^{13}C; guanido-$^{15}N_2$]arginine isotope will allow all aspects of arginine metabolism to be examined. The use of this isotope would allow the amount of arginine being metabolized by the arginase and nitric oxide synthase pathways to be quantified, including the rate of arginine recycling back to arginine through both of these pathways. The rate of oxidative arginine catabolism could also be simultaneously measured due to the 1-^{13}C label. Furthermore, the appearance of the label in intestinal protein would give an indication of the amount of the extracted dietary arginine that is used for protein synthesis. If the [1-^{13}C; guanido-$^{15}N_2$]arginine isotope was administered in combination with gabaculine administration, an inhibitor of OAT (16) and therefore of oxidative arginine catabolism via the citric acid cycle, and the rate of $^{13}CO_2$ production was measured, then the use of arginine in polyamine synthesis could be assessed. This approach would provide an estimate of polyamine synthesis because when ornithine is converted to putrescine, carbon dioxide is released in a 1:1 molar proportion. Alternatively, the use of arginine in polyamine synthesis could be measured more directly using an arginine isotope with the label associated with either the amino-nitrogen or carbons 2 through 5 and measuring conversion rates. The infusion of the arginine isotope by all three routes of infusion, intragastrically, intravenously and intraportally, will allow the roles of first-pass intestinal and hepatic metabolism in the metabolism of arginine by the various pathways to be quantified.

8.4 Conclusion

In conclusion, the series of studies described in this thesis have contributed to the knowledge of tissue sites and limiting factors for arginine synthesis in neonates, and have also looked at other aspects of arginine metabolism including nitric oxide synthesis and splanchnic arginine metabolism. Enteral nutrition is critical for maximum rates of arginine synthesis; therefore, in parenterally-fed neonates it is critical to ensure that the solution provided contains enough arginine to meet the metabolic needs. With the exception of first-pass intestinal metabolism, the other tissue sites involved in arginine synthesis in both parenterally and enterally-fed piglets still require elucidation, although it appears likely that the intestinal metabolism of circulating precursors plays an important role. Despite being capable of greater rates of arginine synthesis than parenterally-fed piglets, enterally-fed piglets have an upper limit to their rate of endogenous synthesis due to a limitation in citrulline formation. Therefore nutritional strategies to increase arginine status in human neonates and piglets could focus on ways to overcome the limitation in citrulline formation. The results from this thesis are an important contribution to our knowledge of neonatal arginine metabolism, specifically in piglet and human neonates where poor arginine status has been associated with impaired performance or health. There are many potential areas for future research with regards to arginine metabolism in neonates.

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